Universidad Nacional de Córdoba. Facultad de Ciencias Médicas.
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FRAGMENTACIÓN DE ADN ESPERMÁTICO EN PACIENTES INFÉRTILES Y SU CORRELACIÓN CON TÉCNICAS DE REPRODUCCIÓN ASISTIDA DE ALTA COMPLEJIDAD (ICSI)

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Director de Tesis:

Prof. Dr. Rubén Daniel Ruiz

Instituto y Cátedra de Fisiología Humana
Facultad de Ciencias Médicas
Universidad Nacional de Córdoba

Co-Director:

Prof. Dr. Sergio Oehninger

Jones Institute for Reproductive Medicine
Director of the Division of Reproductive Endocrinology and Infertility
Professor and Vice Chair of the Department of Obstetrics and Gynecology
Professor of Urology
Eastern Virginia Medical School
Norfolk, Virginia, USA

Comisión de seguimiento:

Prof. Dra. Susana Rubiales de Barioglio

Prof. Dr. Otilio Rosato

Tribunal de Tesis

Prof. Dr Otilio Rosato

Prof. Dr. Carlos Lopez

Prof. Dr. Héctor Gauna

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"LA FACULTAD DE CIENCIAS MEDICAS NO SE HACE SOLIDARIA CON LAS OPINIONES DE ESTA TESIS"

(Art. 43.- Reglamento de la Carrera de Doctorado en Ciencias de la Salud - RHCD 722/2010 y 210/2011; RHCS 1027/2010 y RHCS 327/2011)

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Resumen

La integridad del ADN es crucial para el normal desarrollo embrionario. La evaluación de la fragmentación de ADN espermático se ha propuesto como un método para predecir probabilidades de lograr un embarazo, tanto por concepción natural como por técnicas de reproducción asistida (TRA). La inyección intracitoplasmática del espermatozoide (ICSI) es la TRA de alta complejidad más utilizada en los últimos años. Sin embargo, ésta técnica es mucho más invasiva y no tiene en cuenta las características moleculares o genéticas del espermatozoide seleccionado. Lo cual podría llevar a introducir dentro del óvulo a espermatozoides con ADN dañado.

Mediante la técnica de TUNEL se evaluó la fragmentación de ADN en espermatozoides de hombres infértiles que realizaban TRA por ICSI. Los valores fueron cotejados con los resultados del procedimiento. No se observaron correlaciones ni diferencias significativas entre el porcentaje de espermatozoides con fragmentación de ADN y las tasas de fertilización, calidad embrionaria o tasa de embarazo.

Utilizando la evaluación simultanea de morfología espermática y fragmentación de ADN, se determinó que la mayor proporción de espermatozoides con ADN dañado eran células con morfología espermática anómala. Solo el 1,9 % de los espermatozoides con ADN fragmentado presentaban morfología normal.

En el procedimiento de ICSI solo los espermatozoides móviles y morfológicamente normales son seleccionados para ser inyectados. Se realizó la evaluación de la fragmentación de ADN en espermatozoides morfológicamente normales en muestras usadas para ICSI. Se observó una correlación negativa entre el porcentaje de espermatozoides normales con fragmentación de ADN y calidad embrionaria. Por otro lado, se consiguió encontrar un límite de corte para predecir probabilidades de embarazo.

Se logró aplicar la técnica de TUNEL para la evaluación de fragmentación de ADN en espermatozoides de ratón con lo cual se desarrolló una prueba piloto. Ratones alimentados con una dieta suplementada con omega 3 fueron sacrificados y la integridad del ADN de espermatozoides epididimarios fue comparada con un grupo control. Si bien no se observaron diferencias significativas entre los grupos, estos estudios dan comienzo a la utilización de la técnica que se podrá usar dentro del área de investigación de la Cátedra de Fisiología Humana de la Facultad de Ciencias Médicas de la Universidad Nacional de Córdoba.

Abstract

DNA integrity is crucial to normal embryonic development. Sperm DNA fragmentation evaluation has been proposed as a method to predict pregnancy, both natural conception and assisted reproductive technique (ART). Intracytoplasmatic sperm injection (ICSI) is the ART most used in the last years. However, this technique is most invasive and ignore molecular and genetics condition of selected sperm. This could lead to inject DNA damaged sperm into the egg.

Sperm DNA fragmentation was evaluated by TUNEL in a fraction of the same separated sample used for ICSI. Percentage of sperm DNA damage was compared with the ICSI outcomes. There was not a statistically significant association between percentage of sperm DNA integrity and fertilization, embryo quality or pregnancy outcome.

Simultaneous evaluation of sperm DNA fragmentation and morphology in the same sperm cell was used. Our data indicate that the majority of the cells showing DNA fragmentation have abnormal forms. Only 1.9% of sperm with fragmented DNA showed normal morphology.

It is well known that in ICSI procedure only motile and normal sperm will be selected to injection. According this knowledge and our previous results, sperm DNA fragmentation evaluation was performed in normal spermatozoa from samples used for ICSI. Results demonstrate an association between the incidence of morphologically normal spermatozoa with fragmented DNA and poor embryo quality. In addition, a threshold was found to predict likelihood to pregnancy.

TUNEL assay was setup to evaluate mouse sperm DNA fragmentation and a pilot study was developed. Mice fed a diet supplemented with omega-3 were sacrificed and DNA integrity of epididymal sperm was compared with a control group. No significant differences between groups were found. Nevertheless, these studies allow the use of a new technique that it will be applied in the research area of the Department of Human Physiology, Faculty of Medical Sciences of the National University of Córdoba.

INTRODUCCIÓN

INTRODUCCIÓN

La infertilidad es una enfermedad del sistema reproductivo definida como la incapacidad de lograr un embarazo clínico después de 12 meses o más de relaciones sexuales no protegidas (Zegers-Hochschild et al, 2009). Se estima que aproximadamente en la actualidad 72,4 millones de personas en el mundo tienen problemas para lograr un embarazo y necesitarán atención médica de especialistas en medicina reproductiva (Boivin et al, 2007). Dentro de las parejas infértiles, la gran mayoría logrará el embarazo a través de la inducción de la ovulación o la estimulación ovárica controlada o inseminaciones intrauterinas, intracervicales o intravaginales; mientras que aproximadamente el 20 % necesitará de tratamientos de reproducción asistida de alta complejidad (SAMER, 2012). La fertilización in vitro es la técnica por la cual un ovocito es fecundado por un espermatozoide fuera del cuerpo humano. Las técnicas de fertilización in vitro convencional (FIV) y la inyección de un espermatozoide dentro del citoplasma del ovocito (ICSI) son los tratamientos de alta complejidad más utilizados en la actualidad (CDC, 2012; Ferraretti et al, 2012; SAMER, 2012).

Desde la aparición del ICSI muchos hombres con severa alteración en los parámetros seminales han logrado ser padres biológicos y se ha convertido en la técnica de reproducción asistida de alta complejidad más utilizada en todo el mundo (CDC, 2012; Ferraretti et al, 2012; SAMER, 2012). Este método consiste en la selección e inyección de un solo espermatozoide dentro del citoplasma del ovocito (Palermo et al, 1992). Sin embargo, estas metodologías tienen una eficiencia de entre el 30 % y el 40 % en lograr un embarazo (Ferraretti et al, 2012). Esta "ineficacia" puede estar dada por la utilización de ovocitos con alguna anomalía, fallas en la capacidad receptiva del endometrio o la utilización de espermatozoides con anomalías no detectables con simple observación microscópica.

Durante mucho tiempo se ha propuesto que la fertilización era dependiente de múltiples cualidades inherentes a los ovocitos (Van Blerkom, 2000; Swain y Pool, 2008). Sin embargo, en las últimas dos décadas, diferentes trabajos evaluaron los resultados de la utilización de técnicas de reproducción asistida y proporcionaron el soporte inicial para el concepto de la contribución paterna a la fertilización defectuosa y la embriogénesis anormal. Una fuerte evidencia asocia actualmente la presencia de parámetros espermáticos anormales (principalmente teratozoospermia pero también estrés oxidativo y fragmentación del ADN) con retardada o fallida fertilización y/o

desarrollo embrionario anormal (Kruger et al, 1988; Oehninger et al, 1988; Oehninger et al, 1996; Barroso et al, 2000; Aitken y Krausz, 2001; Tesarik et al, 2004).

Los términos "efectos paternos tempranos o tardíos" han sido propuestos para designar a estas dos condiciones patológicas. La evidencia de un efecto paterno temprano se basa en cigotos de baja calidad y no está asociado con la fragmentación del ADN espermático. El efecto paterno tardío, por el contrario, se manifiesta por un pobre desarrollo embrionario que lleva a una falla en la implantación y se asocia con un aumento en la incidencia de la fragmentación del ADN en ausencia de anormalidades en fase de cigoto y principios de división (Tesarik et al, 2004; Tesarik, 2005).

Se puede especular que los efectos paternos tempranos pueden incluir trastornos espermáticos relacionados con la activación del ovocito y las aberraciones del aparato centrosoma/citoesqueleto. Por otro lado, el efecto paterno tardío se asocia con anormalidades en los espermatozoides a nivel del ADN cromosómico y disfunciones mitocondriales espermáticas.

Para poder comprender e interpretar las diferentes fallas en este proceso, es necesario conocer todos los "participantes" y su función dentro de este sistema.

La gameta femenina: el ovocito

Un ovocito es un óvulo en desarrollo; su diferenciación en un óvulo maduro implica una serie de cambios a partir de células germinales que migran en la gónada en estadíos muy tempranos del desarrollo, transformándose en ovogonias. Tras la proliferación mitótica, las ovogonias se transforman en ovocitos primarios que inician la división meiótica I, y se detienen en la profase. Durante este período, los ovocitos primarios crecen, sintetizan una cubierta y acumulan ribosomas, RNA mensajero y proteínas, a menudo utilizando la ayuda de otras células, como las células accesorias que los rodean. En el proceso de maduración los ovocitos primarios completan la división meiótica I formando un pequeño corpúsculo polar y un gran ovocito secundario, el cual progresa hasta la metafase de la división meiótica II. En esta etapa, el ovocito se detiene hasta que es estimulado mediante su fecundación, completando la meiosis e iniciando el desarrollo embrionario. En el humano, la ovogonia prolifera únicamente en el feto, entra en meiosis después del parto y se detiene en forma de ovocitos en la primera profase meiótica, que puede mantenerse durante más de 50 años.

Los ovocitos maduran en una cantidad estrictamente limitada (cohorte) en cada ciclo y ovulan a intervalos, generalmente uno cada vez, desde la pubertad (Wassarman, 1988). Este proceso es graficado en la figura 1.

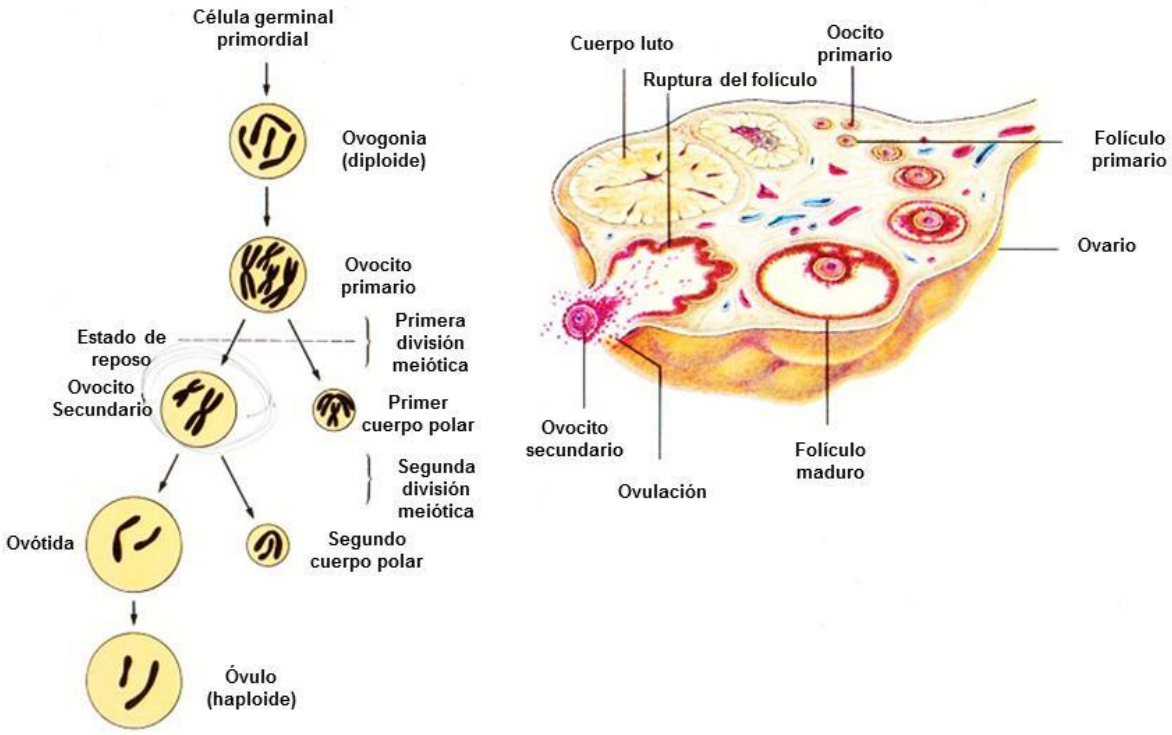


Figura 1. Ovogénesis. *Izquierda: Esquema de la ovogénesis. Derecha: esquema de un corte transversal de un ovario donde se representa la ovogenesis (Tomado de www. http://ldysinger.stjohnsem.edu).*

La gameta masculina: el espermatozoide

Al igual que los ovocitos, los espermatozoides son células altamente especializadas que tienen como función principal transportar el material genético y

fusionarse con el ovocito para crear un nuevo individuo genéticamente diferente a sus progenitores.

El espermatozoide es el producto final del proceso de espermatogénesis a través de sucesivas mitosis, fases meióticas y postmeióticas dentro de los túbulos seminíferos de los testículos. A diferencia de la mujer, la gametogénesis en el hombre no comienza hasta la pubertad y a partir de ese momento se produce de manera continua en la pared epitelial de los túbulos seminíferos. Las espermatogonias, células germinales inmaduras, están situadas alrededor del borde externo de estos tubos, junto a la lámina basal, donde proliferan continuamente por ciclos de división celular ordinaria. Durante la fase de mitosis, en las células germinales ocurre una serie de divisiones que incrementa la población de espermatogonias. La fase meiótica comienza con el último ciclo celular de la fase S y culmina en dos divisiones meióticas, que rápidamente se producen sin la replicación del ADN para producir espermátides haploides. Mientras que estas dos fases son cruciales para el desarrollo del gameto masculino, es durante la fase de post meiosis que los espermatozoides adquieren su morfología flagelada. Esta fase se caracteriza por una amplia remodelación de las espermátides mediante la formación del acrosoma, la condensación nuclear, el desarrollo del flagelo, y la pérdida de la mayor parte del citoplasma. Posteriormente, estas células completan su diferenciación morfológica a espermatozoides en la luz del túbulo seminífero. Luego los espermatozoides migran al epidídimo, un tubo enrollado bilateral situado sobre la superficie testicular, donde sufren la maduración final y son almacenados hasta el momento de la eyaculación. Estos eventos resultan en una célula altamente diferenciada en estructura y función, y capaz de combinarse con un ovocito para comenzar el proceso que da lugar a la próxima generación (Eddy, 1994).

El espermatozoide está formado por tres regiones, morfológica y funcionalmente diferentes entre sí, rodeados por una misma membrana plasmática: la cola, que impulsa al espermatozoide hacia el ovocito y ayuda a penetrarlo; la pieza media, rica en mitocondrias que hidrolizan ATP para generar energía para el movimiento de la cola y la cabeza, que contiene un núcleo haploide altamente condensado (figura 2). El ADN del núcleo se encuentra extremadamente empaquetado, de modo que su volumen esta reducido al mínimo favoreciendo el transporte. Los cromosomas de los espermatozoides humanos (como de otras especies) tienen una disminuida cantidad de histonas y son reemplazadas por nucleoproteínas de elevada carga positiva (ricos en cisteína y arginina) denominadas protaminas (Eddy, 1994).

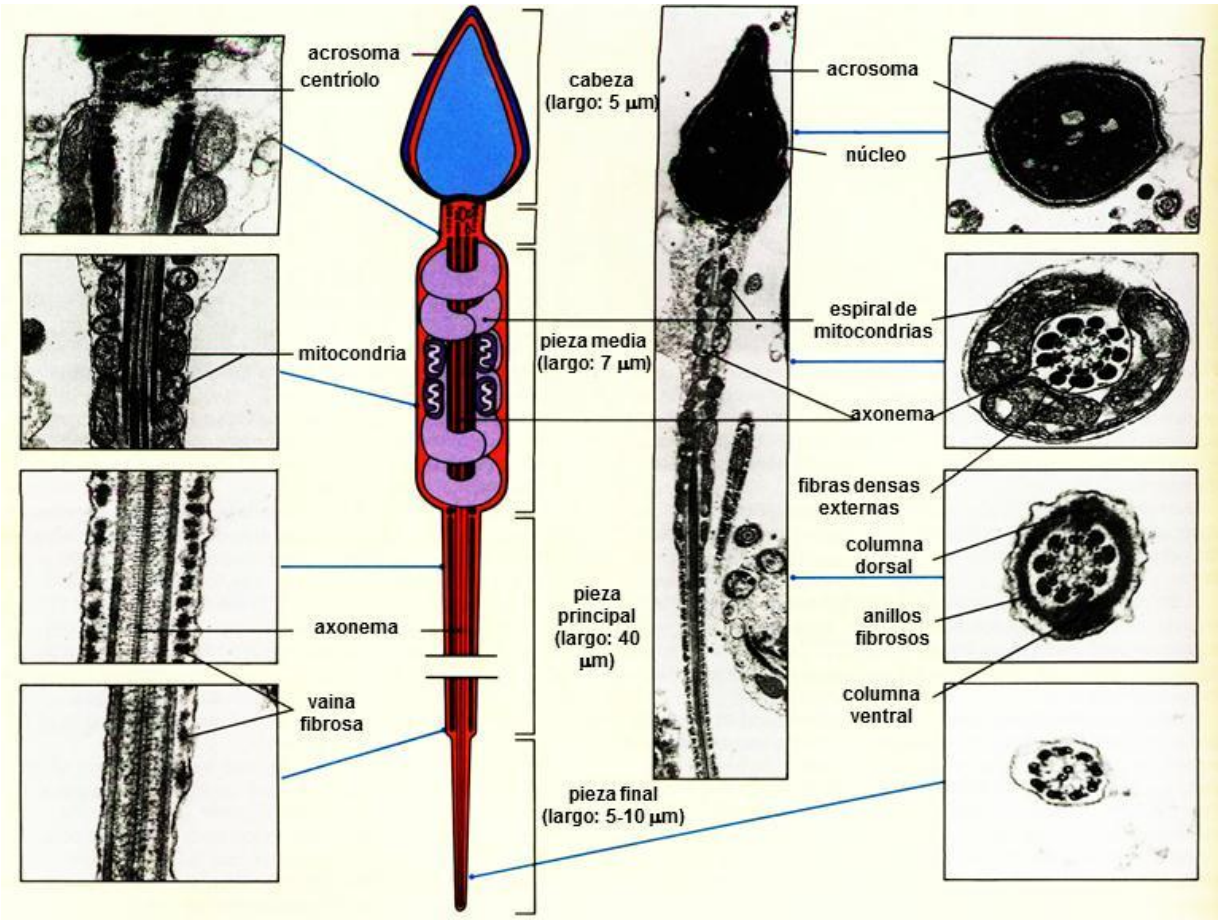


Figura 2. Estructura del espermatozoide maduro. *Representación esquemática y ultramicrofotográfica (izq.: corte longitudinal; der.: corte tranversal) a partir de secciones de las diferentes porciones de la estructura celular (Stevens y Lowe, 1992).*

En la cabeza de los espermatozoides, rodeando el extremo anterior del núcleo, se encuentra una vesícula secretora especializada denominada vesícula acrosomal o acrosoma (figura 2). Esta vesícula contiene enzimas hidrolíticas que ayudan al espermatozoide a atravesar la zona pelúcida que rodea al ovocito. Cuando un espermatozoide entra en contacto con un ovocito, el contenido de la vesícula se libera por exocitosis, denominada reacción acrosómica, que facilitará, por hidrólisis, el paso del espermatozoide para fusionarse con el ovolema.

La fertilización

La fertilización es el proceso mediante el cual dos gametos haploides, el espermatozoide y el ovocito, se unen para producir un individuo genéticamente distinto. Se reconoce que la fertilización es el resultado de una secuencia precisa y ordenada de interacciones celulares y que se inicia con el contacto entre un espermatozoide y un ovocito.

El espermatozoide eyaculado debe experimentar una serie de procesos indispensables para fertilizar al ovocito. Entre ellos podemos mencionar: el proceso de capacitación, reconocer y unirse a la zona pelucida (ZP) y sufrir la reacción acrosomal (RA). Estos procesos son resumidos en la figura 3. Los procesos más significativos que experimentan los espermatozoides durante la capacitación son cambios en la membrana plasmática, aumento de algunos mensajeros intracelulares y aumento de fosforilaciones en los residuos tirosina de las proteínas de la membrana plasmática (Yanagimachi, 1994). Se ha descripto en ratones, que la unión de las gametas es llevada a cabo por la interacción entre la proteína 3 de la ZP (ZP3) y proteínas todavía no caracterizadas presentes en la membrana plasmática del espermatozoide. La ZP3 desencadena la RA, seguida por un proceso secundario de unión con la participación de la proteína 2 de la ZP (ZP2) y la membrana acrosomal interna de los espermatozoides que conlleva a la penetración de la ZP. La glicosilación parece ser un paso obligatorio para la función de ligando de la ZP3 en ratones. Se ha demostrado que la glicosilación del grupo hidroxilo (O-glicosilación), y en particular residuos terminales de galactosa, son esenciales para mantener la interacción entre los gametos (Yanagimachi, 1994).

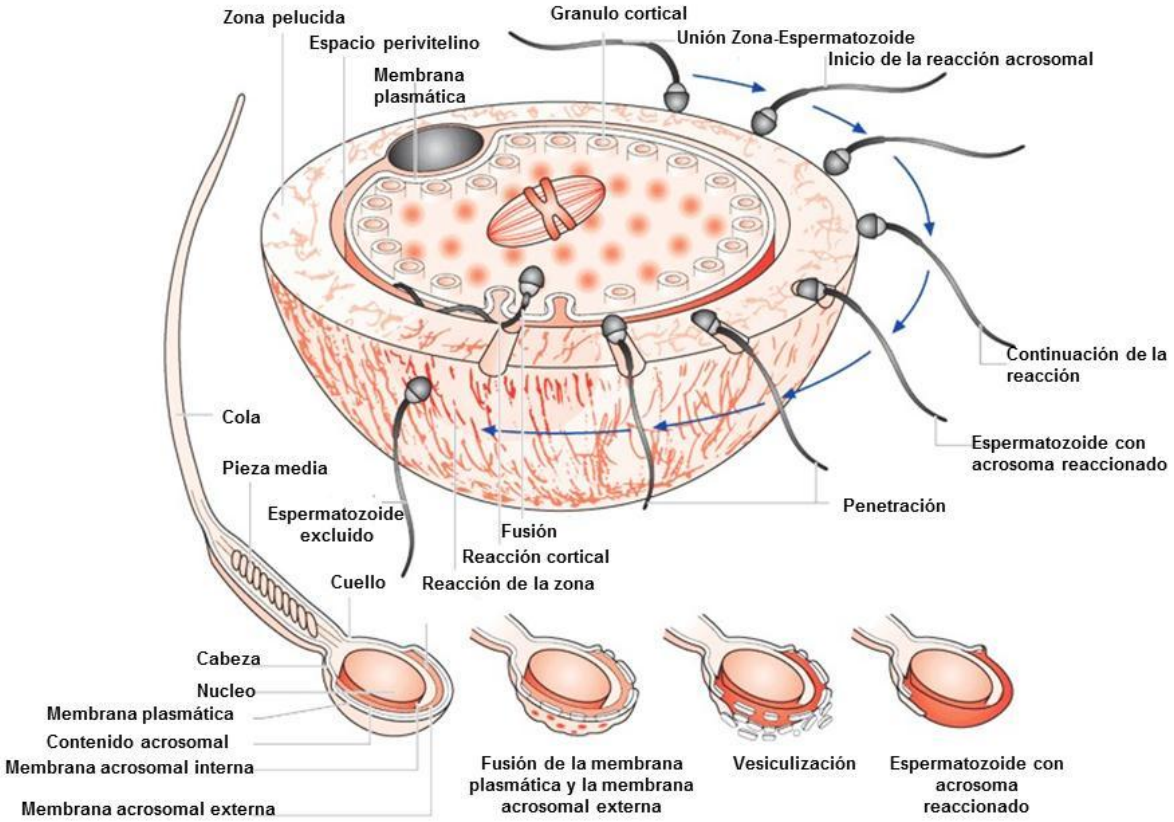


Figura 3. Secuencia de eventos tempranos de la fertilización. *Esquema de las etapas de penetración del espermatozoide a través de la zona pelucida, reacción acrosomal y fusión (Evans et al, 2006)*

Durante las últimas dos décadas, diferentes grupos de investigación han propuesto una variedad de proteínas e hidratos de carbono como posibles receptores de ZP3 en el espermatozoide: 95 kd tirosina-quinasa (Leyton et al, 1992); sp56 (Bookbinder et al, 1995); proteína tipo tripsina (Boettger-Tong et al, 1993); b1-4 galactosiltransferasa (Shur, 1994) y espermadesinas (Gao y Garbers, 1998). Sin embargo, ninguna de estas moléculas ha demostrado ser, de manera inequívoca, un receptor activo y la relevancia fisiológica de estos candidatos es aún objeto de debate.

Durante la fertilización, el espermatozoide con el acrosoma reaccionado se fusiona con el ovolema, y el espermatozoide completo (con la excepción de parte de la membrana plasmática que rodea al acrosoma, la membrana acrosomal externa, y los componentes acrosomales) se incorpora dentro del ovocito (Yanagimachi, 1998).

La unión del espermatozoide a la membrana plasmática del ovocito parecería estar mediada por los miembros de la familia de proteínas secretoras ricas en cisteína (CRISP1 y CRISP2) y miembros de la familia de proteínas la conocidas como las metaloproteasas–desintegrinas (ADAM del inglés A Disintegrin And Metalloprotease) en los espermatozoides y el receptor de la integrina alpha$_{vi}$ß1 de los ovocitos (Snell y White, 1996) . La unión del espermatozoide a la integrina β1 es un prerrequisito para la fusión de las membranas del espermatozoide con el ovolema en el proceso de fertilización en mamíferos (Cowan et al, 2001). La integrina del ovocito es necesaria para la fusión de membranas y su actividad parecería estar relacionada con una proteína de la superficie de los espermatozoides denominado PH-30 que estaría implicada en la fusión de los gametos (Primakoff y Myles, 2000).

Se han propuesto tres mecanismos diferentes por el cual el espermatozoide iniciaría la activación del ovocito: 1) la teoría de la fusión, sugiere que los componentes de la cabeza del espermatozoide activan la liberación de calcio (Dale et al, 1985; Fissore et al, 1999); 2) teoría del receptor, propone una transducción de señales mediada por receptores localizados en la membrana plasmática de ovocitos (Kline et al, 1988); 3) la teoría de la bomba de calcio, que propone que el Ca^{2+} es ingresado en el ovocito o en los depósitos, por el propio espermatozoide a través de canales presentes en la membrana plasmática del espermatozoide (Jaffe, 1983).

La evidencia actual apoya la idea de que un sistema receptor de inositol-3-fosfato (IP3) es el principal mediador de las oscilaciones de calcio en ovocitos (Parrington et al, 1996). Se ha demostrado que un factor soluble del espermatozoide, conocido como fosfolipasa C zeta (PLC zeta), provoca oscilaciones de calcio y la activación del ovocito en los mamíferos (Saunders et al, 2002). Se postula además que luego de la fusión de las membranas plasmáticas del espermatozoide y el ovocito, la PLC zeta del espermatozoide difunde en el citoplasma del ovulo dando como resultado la hidrólisis de PIP2 (fosfatidilinositol 4, 5-bifosfato) de una fuente desconocida para generar IP3 (inositol 1,4,5-trifosfato) (Swann et al, 2004; Saunders et al, 2007).

En los primates, el pronúcleo masculino está fuertemente asociado con el centrosoma, que organiza a los microtúbulos para formar el áster espermático que impulsa al centrosoma del espermatozoide desde la periferia nuclear del ovocito recién fecundado hacia el centro de la gameta. Por el contrario, el pronúcleo femenino humano no tiene centrosoma asociado a los microtúbulos. El actual modelo del movimiento del

pronúcleo femenino implica un desplazamiento a lo largo de la red de microtúbulos (Schatten, 1994; Allan, 1996; Payne et al, 2003).

Si alguno de estos mecanismos falla, la pareja se verá imposibilitada de concebir de forma natural y requerirá de la asistencia de tratamientos de medicina reproductiva. De acuerdo a la bibliografía, se puede especular que entre el 15 al 20 % de parejas en edad reproductiva (de 18 a 44 años, es decir unos 50 a 80 millones de personas en el mundo) han tenido, tienen o tendrán dificultades para lograr un embarazo (Payson y Armstrong, 2007) y algunas de ellas deberán someterse a tratamientos de reproducción asistida. Alrededor del 30 % de estas parejas son infértiles debido a un factor masculino aislado, mientras que un 20 % de los casos está asociado a factores masculinos y femeninos coexistentes. Por lo tanto, se podría decir que la infertilidad está causada en un 50 % por un factor masculino (Irvine, 1998).

Técnicas de Reproducción Asistida

Muchas parejas con problemas para concebir son tratadas con métodos de reproducción asistida de alta complejidad, conocida como fertilización *in vitro* (FIV). La FIV es el proceso por el cual un ovocitoes fertilizado por un espermatozoide fuera del cuerpo humano (*in vitro*). El proceso implica el monitoreo del proceso ovulatorio de la mujer, la obtención de los ovocitos de los ovarios por medio de microcirugía e incubación en un medio de cultivo con espermatozoides.

Desde el nacimiento del Louise Brown, el primer bebe nacido por FIV, en 1978 (Steptoe y Edwards, 1978), la FIV se ha convertido en un tratamiendo rutinariamente utilizado en ciertos tipos de infertilidad, incluyendo enfermedades tubarias, endometriosis, infertilidad inexplicable o infertilidad por factor masculino leve. Sin embargo, la escasa cantidad de espermatozoides, deficiencia en la movilidad y/o pobre morfología espermática, representa la principal causa de fallas en la fertilización por medio de la FIV convencional. Con la incorporación de la inyección intracitoplasmatica de un espermatozoide (ICSI) se han resuelto muchos de estos inconvenientes (Palermo et al, 1992). La técnica de ICSI difiere de la FIV convencional en que en este caso un solo espermatozoide móvil y de aspecto morfológicamente normal es inyectado directamente dentro del ovoplasma (Figura 4).

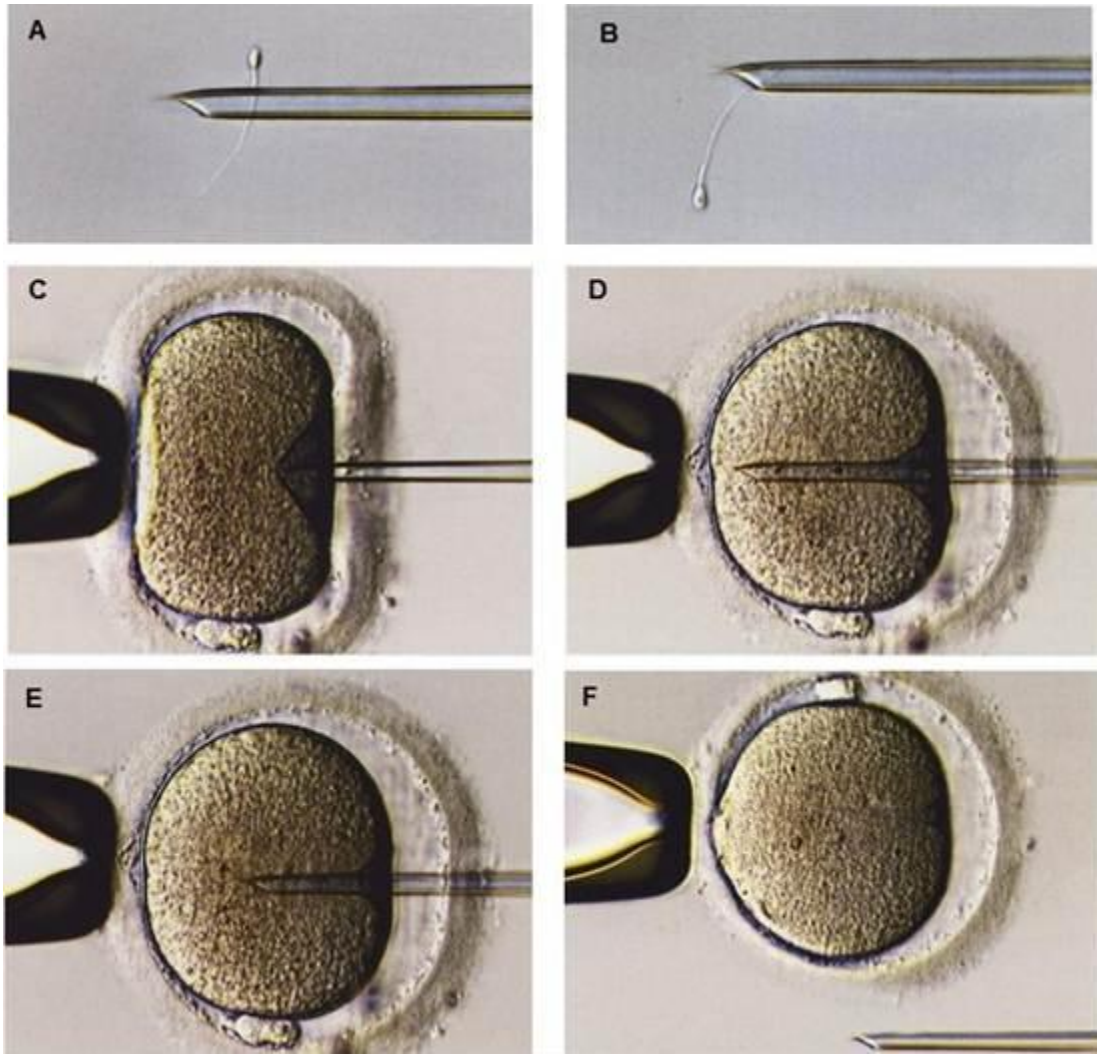


Figura 4. Pasos del procedimiento de ICSI*. A: inmovilización del espermatozoide; B: aspiración del espermatozoide dentro de la pipeta de inyección; C: penetración a través de la zona pelucida; D: inyección de la pipeta dentro del ovoplasma; E: deposito del espermatozoide dentro del oolema; F: una vez depositado el espermatozoide, la pipeta es removida lentamente del oolema (Veeck, 1999)*

Sin embargo si el material genético del espermatozoide se encuentra dañado, el proceso de fertilización y desarrollo embrionario podría fallar. El espermatozoide podrá fertilizar al ovocito pero podría producir embriones de mala calidad, lo cual conllevaría a bajas probabilidades de lograr un embarazo. En la figura 5 se esquematiza una fertilización y desarrollo embrionario normal. En la figura 6 se muestran diferentes embriones con diferente calidad.

Figura 5. Desarrollo embrionario in vitro*. A, B, C y D: 16 h, 24 h, 48 hs y 72 h pos inseminación, respectivamente. (Veeck, 1999).*

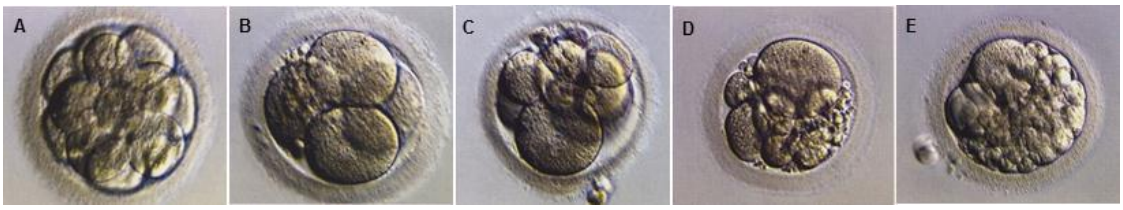


Figura 6. Calidad embrionaria*. Fotomicrografía de embriones humanos 72 h post inseminación. La calidad embrionaria se estableció de acuerdo a la morfología de las gamentas. Se definió grado 1 como el de mejor calidad y grado 5 como el de peor calidad y pronóstico. A: grado 1; B: grado 2; C: grado 3; D: grado 4; E: grado 5 (Veeck, 1999).*

Fallas en la embriogénesis consecuencia de anomalías en la cromatina espermática

Fragmentación del ADN espermático

La función principal del espermatozoide es transportar el material genético hasta el ovocito para luego fusionarse con los cromosomas femeninos en el momento de la fertilización para generar un nuevo individuo. Si el material genético que lleva el espermatozoide se encuentra dañado, su función se verá alterada con la posibilidad de fallos en la fertilización, el crecimiento embrionario, fallas en la implantación o posiblemente transferencia de material genético dañado a la descendencia (Aitken et al, 2009).

El daño en el ADN de los espermatozoides puede afectar tanto al ADN mitocondrial como al ADN nuclear y puede ser inducido por seis mecanismos principales. Éstos pueden ocurrir ya sea durante la producción o el transporte de los espermatozoides y son: [1] la apoptosis durante el proceso de espermatogénesis [2]; durante la remodelación de la cromatina de los espermatozoides, la cadena de ADN se rompe durante el proceso de espermiogénesis; [3] fragmentación del ADN post-testicular inducida principalmente por los radicales libres, incluyendo los radicales hidroxilo, el óxido nítrico y el anión superóxido y durante el transporte de los espermatozoides a través de los túbulos seminíferos y el epidídimo, [4] la fragmentación del ADN inducido por caspasas y endonucleasas endógenas; [5] daño del ADN inducido por radioterapia y quimioterapia y [6] daño del ADN inducido por tóxicos ambientales (Sakkas y Alvarez, 2010) (Figura 7).

De estos seis mecanismos, el que puede jugar un papel importante en causar fragmentación del ADN espermático es el daño post-testicular durante el transporte de los espermatozoides a través del epidídimo. Esto se apoya en los resultados que demuestran que la fragmentación del ADN es mayor en espermatozoides del epidídimo caudal y del eyaculado (Ollero et al, 2001; Greco et al, 2005; Suganuma et al, 2005) en comparación con espermatozoides testiculares.

Durante el proceso de la espermatogénesis, un 50% a 60% de todas las células germinales que entran en meiosis I son inducidas a la apoptosis por medio de un sistema de selección regulado por las células de Sertoli. Estas células son fagocitadas y eliminadas por las células de Sertoli asociadas a ellas, por medio de marcadores apoptóticos como la proteína de transmembrana de tipo II (Fas) perteneciente a la familia del factor de necrosis tumoral (Billig et al, 1996; Pentikainen et al, 1999; Sakkas et al, 1999). Sin embargo, este mecanismo no siempre opera de manera eficiente y un porcentaje variable de células germinales defectuosas evaden esta selección y pueden aparecer más tarde en la eyaculación. Burrello y colaboradores (Burrello et al, 2004), han sugerido que existe una disociación entre la calidad genómica en las células germinales y la remodelación del espermatozoide que se produce durante el proceso de espermiogénesis. Es decir, que una célula germinal puede tener su núcleo alterado por apoptosis o aneuploide y aun así, el espermatozoide resultante tendrá una morfología normal.

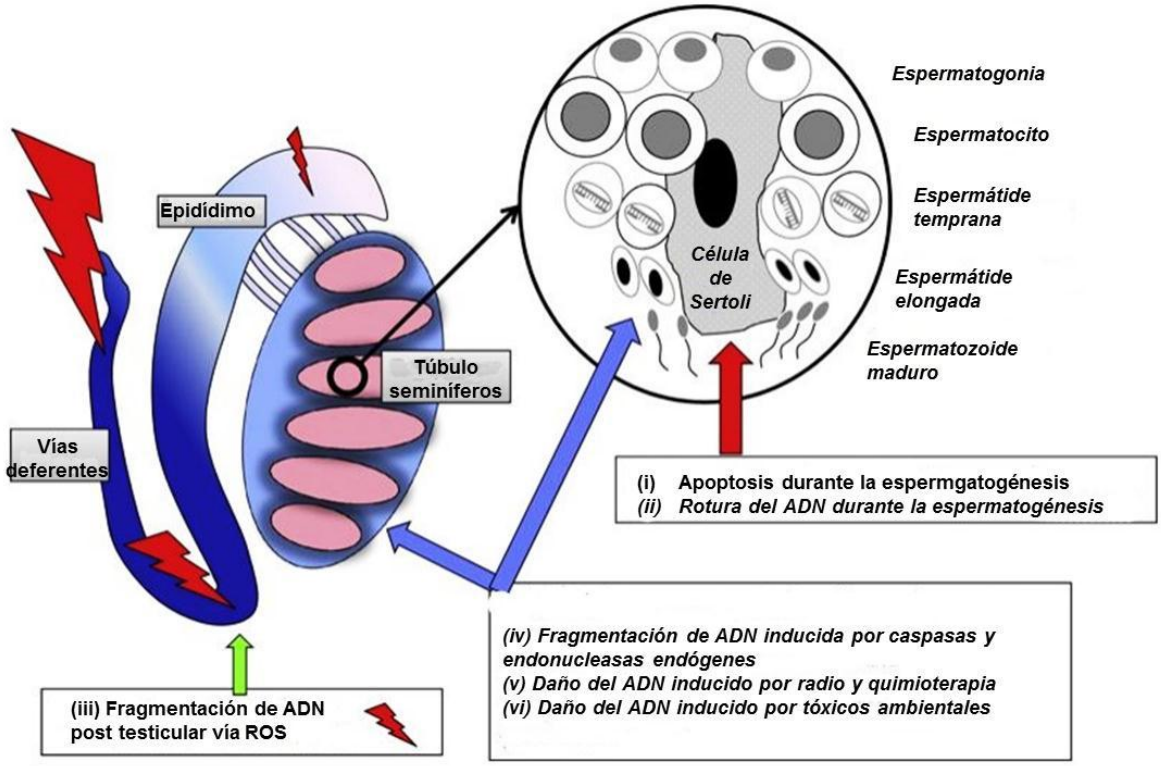


Figura 7. Mecanismos de inducción de daño en el ADN en los espermatozoides*. El daño en el ADN puede ser producido tanto durante la producción o el transporte de los espermatozoides: (i) apoptosis durante el proceso de la espermatogénesis, (ii) las hebras del ADN se rompen durante la remodelación de la cromatina de los espermatozoides durante el proceso de espermiogénesis, (iii) fragmentación del ADN post-testicular inducida principalmente por los radicales de oxígeno (ROS), durante el transporte de los espermatozoides a través de los túbulos seminíferos y el epidídimo, (iv) la fragmentación del ADN inducido por caspasas endógenas, endonucleasas, etc, (v) el daño del ADN inducido por la radioterapia y la quimioterapia, y (vi) el daño del ADN inducido por tóxicos ambientales (Sakkas y Alvarez, 2010).*

Se ha demostrado que en los hombres con oligozoospermia, la probabilidad de un espermatozoide de ser aneuploides con morfología normal es mucho mayor que cuando el hombre es normozoospérmico (Burrello et al, 2004). Esto está probablemente relacionado con la detención parcial de la maduración asociada con alteraciones meióticas. Por otro lado, se ha demostrado que un porcentaje variable de espermatozoides eyaculados expresan marcadores de apoptosis como por ejemplo Fas, fosfatidilserina, Bcl-XL, p53 (Sakkas et al, 2002; Oehninger et al, 2003; Cayli et al, 2004; Mahfouz et al, 2009), lo cual podría sugerir la presencia de mecanismos de apoptosis

similares a los que ocurren en células somáticas. Sin embargo, esto último ha sido recientemente cuestionado (McVicar et al, 2004; Koppers et al, 2011) .

Fragmentación del ADN durante el proceso de espermiogénesis

Las alteraciones en la remodelación de la cromatina durante el proceso de espermiogénesis podrían resultar en la fragmentación del ADN. McPherson y Longo (McPherson y Longo, 1993) postularon que la presencia en espermatozoides eyaculados de roturas del ADN podría ser indicativa de la maduración incompleta durante la espermiogénesis. Ellos indicaron que el empaquetamiento de la cromatina puede requerir la actividad nucleasa endógena para producir y ligar fracturas que faciliten la protaminación. Se especula que estas fracturas del ADN se producen para proporcionar alivio en la disposición de la cromatina durante la sustitución de las histonas por las protaminas (Marcon y Boissonneault, 2004). Alteraciones en este proceso podrían dar lugar a la presencia de anomalías de empaquetamiento de la cromatina o roturas sin reparar del ADN. Éstas se podrían producir previo a la espermiación y es probable que estos espermatozoides sean más susceptibles a daños post-testiculares.

Daño post-testicular del ADN

Algunos estudios mostraron que los espermatozoides inmaduros producen altos niveles de especies de oxigeno reactivas (ROS) y pueden inducir daño en el ADN de los espermatozoides maduros. Este daño se produce después de la espermiación y durante la migración de los espermatozoides desde los túbulos seminíferos a los epidídimos (Ollero et al, 2001). La vida media de las ROS es del orden de nanosegundos a microsegundos (Mahfouz et al, 2009); sin embargo, en el epidídimo los espermatozoides se encuentran fuertemente empaquetados lo que facilitaría el daño en el ADN inducido por ROS. Las ROS pueden dañar el ADN del espermatozoide, directa o indirectamente a través de la activación de las caspasas y endonucleasas del espermatozoide. Esto es consistente con el hecho de que la centrifugación de espermatozoides inmaduros (que producen altos niveles de ROS) con espermatozoides maduros resulta en la inducción de fragmentación del ADN en los espermatozoides maduros, porque en estas condiciones los espermatozoides maduros e inmaduros están en contacto cercano (Twigg et al, 1998). Esto también es consecuente con el hecho de que la exposición *in*

vitro de espermatozoides maduros con altos niveles de ROS resultan en importantes daños del ADN (Aitken et al, 1998; Lopes et al, 1998).

Por otro lado, las células epiteliales del epidídimo también podrían desempeñar un papel activo en el daño al ADN inducido por ROS tales como el anión superóxido o el radical hidroxilo (Britan et al, 2006) o por la activación de las caspasas y endonucleasas espermáticas a través de factores físico-químicos tales como altas temperaturas (Banks et al, 2005) o factores ambientales (Rubes et al, 2007). En el primer caso, el daño en teoría se podría evitar con el uso de antioxidantes, mientras que en el segundo caso, este tratamiento no sería efectivo. Esto es apoyado por el estudio de Greco y colaboradores (Greco et al, 2005) en el que el uso de antioxidantes dio como resultado una reducción significativa en los niveles de fragmentación del ADN espermático.

Cabe señalar que la fragmentación del ADN espermático inducido por la radiación ionizante y por radicales hidroxilo da como resultado la formación de 8-OH-guanina y 8-OH-2'-desoxiguanosina (8-OHdG) en una primera etapa y luego, como consecuencia, la fragmentación del ADN de cadena simple (Cui et al, 2000). Además, la formación de radicales hidroxilo puede dar lugar a la inducción del daño de la doble cadena en el ADN del espermatozoide a través de la activación de las caspasas y endonucleasas.

Métodos para la evaluación de la fragmentación del ADN

Durante las últimas dos décadas una serie de pruebas se han introducido para el análisis de la fragmentación del ADN espermático. Dentro de éstas técnicas se pueden mencionar: el Terminal Uridine Nick-end Labeling (TUNEL) (Gorczyca et al, 1993), el ensayo de cometa (Hughes et al, 1996), Cromomicina A3 (CMA3) (Manicardi et al, 1995), in-situ nick translation (Bianchi et al, 1993; Tomlinson et al, 2001), DNA breakage detection-Fluorescence in situ Hybridization (DBD-FISH) (Fernandez et al, 2000) , la prueba dispersión de la cromatina espermática (SCD) (Fernandez et al, 2003), y el Sperm Chromatin structure assay (SCSA) (Evenson et al, 1980; Evenson et al, 1980; Larson et al, 2000; Evenson et al, 2002).

Un aspecto importante en el análisis de fragmentación del ADN está relacionado con el tipo de roturas producidos en las cadenas de ADN, es decir si las roturas son de

simple o de doble cadena y si se requiere un paso inicial de desnaturalización a fin de detectar roturas en el ADN, como en el caso del SCSA, SCD (Fernandez et al, 2003), o del ensayo del cometa, con un pH ácido o alcalino (Singh et al, 1989). De hecho, cuando el daño del ADN se observa bajo condiciones ácidas o alcalinas y no bajo condiciones de pH neutro, se podría hablar de sitios lábiles ácido/alcalino del ADN (Wyrobek et al, 2006). Por otro lado, el TUNEL, el ISNT y el cometa con un pH neutro (Singh et al, 1989) no requieren de una etapa inicial de desnaturalización y por lo tanto, la evaluación de la rotura de la cadena simple (ISNT, TUNEL, y cometa) o de la doble hebra (TUNEL y cometa) se realiza directamente.

Por lo tanto, estas técnicas podrían agruparse en: [1] pruebas directas en las que se mide el daño del ADN sin un tratamiento previo: TUNEL, ISNT y ensayo de cometa con un pH neutro y [2] las pruebas indirectas que miden el daño del ADN después de la desnaturalización: SCSA, SCD y cometa a pH ácido o alcalino.

Las técnicas de mayor uso clínico en la actualidad son:

SCSA: mide la susceptibilidad del ADN espermático a desnaturalizarse *in situ* inducido por calor o ácido, seguido por la tinción con naranja de acridina (Evenson y Jost, 2000). La detección se realiza por citometría de flujo que permite medir una gran cantidad de espermatozoides por muestra lo que la hace altamente reproducible (Evenson et al, 2002). El naranja de acridina es un marcador metacromático que fluoresce rojo cuando se une con el ADN desnaturalizado (simple hebra) y verde cuando se une al ADN no desnaturalizado (doble hebra). El ADN de los espermatozoides con una estructura normal de la cromatina no se desnaturalizaría mientras que si las cadenas de ADN contienen roturas puede alcanzar diferentes grados de desnaturalización. El SCSA mide diferentes parámetros. El índice de fragmentación del ADN (DFI) que representa la fracción de espermatozoides con ADN de simple cadena. El ADN altamente teñido (HDS) detecta la fracción de espermatozoides con mayor accesibilidad a la tinción metacromática al ADN de doble cadena principalmente debido a la sustitución de las histonas por protaminas. Algunos estudios han indicado que valores de DFI mayores a 27% están asociados con el fracaso del embarazo en tratamientos de reproducción asistida (TRA o ART, del inglés *assisted reproductive techniques*) (Larson et al, 2000; Larson-Cook et al, 2003).

SCD: el ensayo de dispersión de la cromatina espermática (SCD) se basa, al igual que el SCSA, en la susceptibilidad del ADN a descondensarse inducida por un medio

ácido y la evaluación de la dispersión de ADN en halos de tamaño variable (Fernandez et al, 2003). En el último tiempo se ha desarrollado una versión mejorada y estandarizada del protocolo de SCD conocido como Halosperm® (halotech DNA SL, España), con mejor coloración de la cromatina y la preservación de la calidad de la cola (Fernandez et al, 2005). Por ser una técnica nueva, su utilización no está aún muy difundida. Sin embargo, recientemente se ha demostrado su valor en la predicción de la tasa de fertilización, la calidad del embrión, y la tasa de implantación en 85 parejas participantes en un programa de FIV/ICSI (Muriel et al, 2006).

Ensayo de cometa. La electroforesis de una sola célula en un microgel o el ensayo de cometa fue desarrollado para evaluar la integridad del ADN, incluyendo dobles y simples roturas en células somáticas (Ostling y Johanson, 1984). El tratamiento del ADN en condiciones alcalinas (pH > 13) permite la detección de rupturas de cadenas simples de ADN. En este ensayo, el ADN dañado migra en el gel de agarosa y dependiendo de la cantidad de fragmentos de ADN, se creará una cola (similar a un cometa) más grande o más chica, que se visualiza por medio de tinciones específicas para el ADN, mientras que si el ADN está intacto y súper enrollado, no se formara el "cometa".

TUNEL: el ensayo de TUNEL ha sido extensamente usado para la evaluación directa de la fragmentación de ADN espermático. Ésta técnica se basa en la adición de nucleótidos marcados en la porción 3' -OH del ADN dañado o fragmentado por medio de una reacción catalizada por la enzima deoxinucleotidasa terminal transferasa (Gorczyca et al, 1993). La proporción de ADN dañado puede ser medida tanto por microscopía como por citometría de flujo. El ensayo de TUNEL puede detectar tanto el daño de hebra simple como doble. Es importante señalar que aunque la prueba de TUNEL se utiliza con frecuencia para la determinación de la apoptosis celular, un resultado de TUNEL positivo no es sinónimo de apoptosis, como es el caso del daño del ADN inducido por radiaciones ionizantes o por radical hidroxilo (Bianchi et al, 1993). El concepto de que la evaluación del ADN en espermatozoides es asociado a apoptosis es una gran sobreestimación de la capacidad de la prueba. El ensayo de TUNEL tiene baja variabilidad tanto intra como inter observador (Barroso et al, 2000). Debido a su alta especificidad y reproducibilidad, este ensayo es uno de los más frecuentemente usados para evaluar la fragmentación de ADN espermático. Su relación con la función espermática como con la fertilización o embarazo han sido demostrado por diferentes autores (Sun et al, 1997; Oosterhuis et al, 2000; Younglai et al, 2001; Duran et al, 2002; Shen et al, 2002; Benchaib et al, 2003; Henkel et al, 2004).

Impacto de la fragmentación de ADN espermático en la fertilización y el embarazo

Actualmente, no hay dudas de que el daño del ADN espermático puede producir fallas en la fertilización y el inicio del embarazo y también podría provocar efectos perjudiciales sobre la salud de la descendencia (In't Veld et al, 1995; Ji et al, 1997; Kurinczuk y Bower, 1997; Aitken et al, 1998; Twigg et al, 1998; Aitken y Krausz, 2001; Aitken y Sawyer, 2003; Alvarez, 2003). Esto se ha visto reflejado principalmente por ensayos en animales de experimentación donde, la utilización de espermatozoides con ADN dañado provocó embriones de mala calidad, disminución de crías nacidas vivas y hasta podría generar efectos que sólo surgen durante la vida adulta, como el crecimiento aberrante, envejecimiento prematuro, alteraciones del comportamiento y tumores mesenquimales (Fatehi et al, 2006; Fernández-González et al, 2008).

Asimismo, el impacto negativo de la utilización de espermatozoides con ADN dañado en técnicas de reproducción asistida, ha sido demostrado tanto para la inseminación intrauterina (IIU) (Duran et al, 2002) como para la fertilización in vitro (FIV) (Sun et al, 1997; Host et al, 2000; Morris et al, 2002; Henkel et al, 2004) .

Los datos obtenidos por Twigg y col (Twigg et al, 1998) y Henkel y col (Henkel et al, 2003; Henkel et al, 2004) sugieren que los espermatozoides con ADN fragmentado son capaces de fertilizar un ovocito, pero fallan en el desarrollo embrionario lo que resulta en una pérdida temprana del embarazo. Incluso en la concepción natural, el daño oxidativo del ADN del espermatozoide tiene un impacto negativo sobre la fertilidad humana y en el embarazo (Spano et al, 2000; Loft et al, 2003). Sin embargo, en el caso de la inyección intracitoplasmática de espermatozoides (ICSI) se han reportado resultados contradictorios. Mientras que un número de diferentes autores han demostrado una influencia negativa y significativa de los espermatozoides con ADN dañado en la fertilización y el embarazo (Lopes et al, 1998; Henkel et al, 2003; Tesarik et al, 2004), otros no han detectado efecto deletéreo alguno (Host et al, 2000; Gandini et al, 2004; Bungum et al, 2007).

Estas diferencias podrían deberse a que estos estudios presentan variaciones en el diseño experimental, los criterios de exclusión/inclusión o la metodología para evaluar la fragmentación del ADN espermático. Asimismo, podrían ser explicadas por el hecho que durante el procedimiento de ICSI la selección espermática se basa en la movilidad y la aparente normal morfología y no en la selección de una célula al azar.

Por otro lado, debido a que para estudiar la integridad del ADN es necesario fijar y permeabilizar a los espermatozoides, lo cual los hace inviables para su posterior utilización en una técnica de reproducción asistida, la fragmentación del ADN del espermatozoide seleccionado para ICSI no puede ser evaluado. Se ha propuesto que el estudio de la integridad del ADN espermático en muestras de pacientes previo a tratamientos de reproducción asistida sería una buena aproximación a los fines de poder predecir indirectamente las probabilidades de lograr un embarazo (Lewis et al, 2008).

Teniendo en cuenta los antecedentes anteriormente mencionados y considerando la importancia de la integridad del ADN espermático en el proceso de fertilización y el normal desarrollo embrionario, es de vital interés estudiar la integridad del ADN en espermatozoides que serán usados en técnicas de reproducción asistida para de esta forma poder predecir las chances de éxito en parejas que encaran un tratamiento de reproducción asistida de alta complejidad, como es el caso del ICSI.

Aplicación de la técnica de TUNEL en espermatozoides de ratón

El uso de animales de laboratorio en las investigaciones biomédicas representa un elemento fundamental en el desarrollo de importantes avances en la prevención y tratamiento de las enfermedades. El Laboratorio de Reproducción de la Cátedra de Fisiología Humana de la Facultad de Ciencias Médicas de la Universidad Nacional de Córdoba desarrolla en la actualidad un programa dedicado al estudio de factores epigenéticos (nutricionales, farmacológicos y ambientales) sobre la salud reproductiva de mamíferos. Dado que existen al presente pocos trabajos en los cuales se haya explorado la fragmentación de ADN en espermatozoides de ratón (Fernández-González et al, 2008; Oliveira et al, 2009; Bakos et al, 2011) y el interés en la posible aplicación de esta técnica para estudiar la actividad funcional de los gametos, nos propusimos realizar experimentos preliminares aplicando la técnica de TUNEL, de acuerdo a la disponibilidad de recursos financieros y/o de lotes experimentales y sus respectivos controles.

OBJETIVOS

Este trabajo de tesis tiene como objetivo fundamental el estudio del daño del ADN espermático y su posible impacto en los procesos posteriores a la fertilización luego de técnicas de reproducción asistida de alta complejidad (ICSI). Para esto nos propusimos los siguientes objetivos específicos:

- Evaluar el porcentaje de espermatozoides con ADN dañado de pacientes con diagnóstico de infertilidad masculina que serán tratados con ICSI utilizando el ensayo de TUNEL.

- Determinar la incidencia de la fragmentación del ADN en espermatozoides móviles en diferentes subpoblaciones morfológicas.

- Evaluar el porcentaje de espermatozoides normales con fragmentación de ADN en pacientes infértiles con diagnóstico de factor masculino ya que éstos son las células que tienen mayor probabilidad de ser seleccionadas en el momento de la inyección del ovocito en el ICSI.

- Correlacionar la presencia de fragmentación de ADN espermático con resultados del procedimiento de ICSI (fertilización, clivaje, calidad embrionaria y embarazo).

- Validar la técnica de TUNEL para evaluación de fragmentación de ADN en espermatozoides de ratón.

MATERIALES Y MÉTODOS

MATERIALES Y MÉTODOS

Diseño experimental

Recaudos bioéticos: Este estudio fue aprobado por la Comisión de Revisión Institucional de la *Eastern Virginia Medical School* (IRB - EVMS) y todos los participantes firmaron un consentimiento para la utilización de las muestras. Las muestras de semen fueron clasificados en tres grupos:

-Muestras de **hombres fértiles** obtenidas de donantes voluntarios sanos sin ningún tipo de historia de infertilidad y que sus parejas habían concebido y dado a luz a niños en los últimos 2 años.

-Muestras de **pacientes subfértiles** obtenidas de hombres que consultan por infertilidad definida como la incapacidad de lograr un embarazo por lo menos durante un período de 1 año (Gnoth et al, 2005) pero sin diagnóstico certero.

-Muestras de **pacientes infértiles** incluidos en el programa de ICSI del *Jones Institute for Reproductive Medicine* (Norfolk, Virgina, USA) que presentaban diagnóstico de infertilidad de causa masculina, en ausencia de factores femeninos y que no habían logrado un embarazo luego de hiperestimulación ovárica controlada en combinación con la terapia de inseminación intrauterina (Oehninger, 2001).

Todas las personas incluidas en este estudio tenían un examen físico normal, los testículos presentaban volumen normal, ausencia de varicocele y cultivos de semen negativo.

La indicación de ICSI fue realizada a partir del diagnóstico de infertilidad de causa masculina basada en los hallazgos clínicos, los resultados de, por lo menos, un análisis de semen con parámetros espermáticos alterados de acuerdo a los criterios de la Organización Mundial de la Salud (OMS) (WHO, 1999) y/o la incapacidad de lograr un embarazo después más de 2 ciclos de hiperestimulación ovárica controlada en combinación con terapia de inseminación intrauterina en casos con más de 5 millones de espermatozoides móviles totales. Las parejas con factores etiológicos femeninos y con menos de cuatro ovocitos maduros recuperados fueron excluidas del estudio.

Los embarazos clínicos fueron confirmados por la determinación de la sub unidad beta de la hormona Gonadotrofina Coriónica Humana (β-hCG) y por la visualización ecográfica del saco gestacional a las 7 semanas.

Recolección y preparación de las muestras de semen

Las muestras de semen fueron recolectadas por masturbación dentro de frascos estériles luego de 2 a 4 días de abstinencia sexual y dejadas a temperatura ambiente por 30 minutos para permitir la total licuefacción. Luego se evaluaron las características seminales: la concentración y la movilidad espermática fueron cuantificadas con una cámara de Makler (MidAtlantic Diagnostics Inc., Mount Laurel, NJ, USA) mediante el uso de un sistema computarizado de evaluación (HTR-IVOS, Hamilton Thorne Research, Beverly, MA) y fueron manualmente controlados con parámetros fijos de acuerdo a las especificaciones publicadas por Oehninger y col (Oehninger et al, 1990). La morfología fue evaluada por microscopía bajo aceite de inmersión (1000x), usando criterio estricto (Kruger et al, 1986; Kruger et al, 1988; Menkveld et al, 1990) luego de la tinción con STAT III Andrology Stain (MidAtlantic Diagnostics Inc.).

Se seleccionaron espermatozoides móviles mediante la técnica de *swim-up* utilizando medio de cultivo *Fluido tubárico humano* (HTF, Irvine Scientific, Santa Ana, CA) suplementado con 0,2 % de *Albumina sérica humana* (HSA, Irvine Scientific, Santa Ana, CA). Para separar el plasma seminal, los espermatozoides fueron lavados dos veces con HTF-HSA por medio de 10 min de centrifugación a 300 g. Luego del segundo lavado, el sobrenadante fue removido y se colocó HTF-HSA sobre el sedimento y fue incubado durante 60 min a 37 ^{o}C en estufa con 5 % CO_2. Para recuperar la fracción con mayor movilidad, se aspiró solo la parte más superficial del medio. Luego, los espermatozoides fueron resuspendidos en HTF-HSA y la concentración fue ajustada entre 5 y 10 millones de gametas por mililitro y almacenados hasta su utilización a -196 ^{o}C sin añadir criopreservantes.

Las muestras fueron descongeladas en baño de agua a 37 ^{o}C durante 3 minutos inmediatamente antes de la evaluación de la fragmentación de ADN y la morfología. Una alícuota de aproximadamente 25 µl fue transferida a un portaobjetos multi pocillos (Cell-Line/Erie, Scientific Co, Portsmouth, NH) para la cuantificación simultánea de fragmentación de ADN y morfología mediante microscopía de inmunofluorescencia y

contraste de fase, respectivamente. Cada muestra fue analizada por duplicado y los resultados fueron promediados.

Análisis de semen

El análisis de semen fue llevado a cabo por el Laboratorio de Andrología del *Jones Institute for Reproductive Medicine* de acuerdo a las recomendaciones de la Organización Mundial de la Salud (OMS) (WHO, 1999). Luego de la completa licuefacción del semen se realizó el examen físico-químico (pH, volumen) y microscópico (concentración, movilidad y morfología) del eyaculado.

La morfología espermática fue determinada utilizando el criterio estricto desarrollado por Kruger y col (Kruger et al, 1986) y expresado como porcentaje de espermatozoides normales. Las muestras fueron rotuladas con un número de manera que la identidad del participante permanezca anónima.

A continuación se indican los valores de referencia establecidos por la OMS (WHO, 1999) para los parámetros de concentración, movilidad y morfología espermática: concentración espermática en el semen ≥ $20x10^6$ espermatozoides/ml; movilidad progresiva ≥ 50 %; morfología normal ≥ 15 %. De acuerdo a cuál de estos parámetros se encuentre por debajo de los valores indicados, las muestras de semen se designan de acuerdo a la tabla 1 (WHO, 1999).

Tabla 1. Nomenclatura de algunas variables del semen

Nomenclatura	Definición
Normozoospermia	Eyaculado normal dentro de los valores de referencia
Oligozoospermia	Concentración < 20 x 10^6 / mL
Astenozoospermia	Movilidad < a 50 % de espermatozoides progresivos
Teratozoospermia	Morfología ≤ 14 % de espermatozoides normales
Oligoastenoteratozoospermia	Perturbación significativa de las tres variables
Azoospermia	Ausencia de espermatozoides en el eyaculado
Hipospermia	Disminución del volumen eyaculado

Estimulación ovárica

Para obtener una respuesta suprafisiológica, aumentando así el número de ovocitos disponibles para fertilizar y como consecuencia, el número de embriones y las posibilidades de embarazo, se realizó una hiperestimulación ovárica controlada de acuerdo a protocolos de estimulación previamente publicados (Oehninger, 2011). Para ello, se administraron a las pacientes altas dosis de FSH (Gonal F® o Menopur®) durante la fase folicular de reclutamiento. El desarrollo folicular se controló a partir de niveles séricos de estradiol (E2) y mediante monitoreo ecográfico y se continuó la estimulación usando un régimen de reducción de gonadotrofinas. Cuando los folículos tenían un diámetro de 17 mm se administró una dosis de hCG para provocar la ovulación. Luego de 34 a 35 horas post administración de hCG se realizó la recolección de ovocitos mediante ecografía transvaginal (Oehninger, 2011).

ICSI y ensayo de la calidad embrionaria

Los ovocitos fueron separados de las células del *cúmulus oophorus* mediante el uso de hialuronidasa 0,1 % preparado en medio HTF suplementado con HSA durante 10 segundos y clasificados de acuerdo a su grado de madurez: Metafase II (MII), Metafase I (MI) y Profase I o Vesícula Germinal (VG) (Veeck, 1999). Los ovocitos maduros, es decir que hayan concluido su primera división meiótica (MII) fueron inyectados. Se inyectó un único espermatozoide móvil y con morfología normal dentro del citoplasma del ovocito con ayuda de una pipeta de vidrio extremadamente fina (diámetro 5-6 μm) bajo control microscópico (60x).

Dieciséis a 20 horas luego de la inyección, los ovocitos fueron evaluados. La presencia de dos pronúcleos indicó normal fertilización diploide. Setenta y dos horas después de la recuperación de los ovocitos, los embriones fueron clasificados de acuerdo a su calidad (número y morfología de las blastómeras) y luego los embriones de mejor calidad fueron transferidos al útero bajo control mediante ecografía abdominal. La morfología de los embriones fue registrada en grados de acuerdo al criterio establecido por Veeck ((Veeck, 1999) con las siguientes modificaciones: **Grado 5**: todas las blastómeras de igual tamaño y sin fragmentación; **Grado 4**: blastómeras con igual tamaño y con una fragmentación menor al 20 %; **Grado 3**: blastómeras con diferentes tamaños sin fragmentaciones; **Grado 2**: blastómeras con diferentes tamaños y

más del 20% del volumen fragmentado y **Grado 1**: más del 50% fragmentado (Figura 5). La calidad de cada embrión fue dada por un valor numérico calculado multiplicando el número de blastómeras por el grado de morfología.

CE= Blast x Morfo

CE= calidad embrionaria;
Blast= número de blastómeras;
Morfo= grado de morfología embrionaria

Fueron establecidos dos valores para examinar la calidad embrionaria:

a) la media de la calidad embrionaria de todos los embriones de una determinada paciente (CETot) fue calculada como la sumatoria del valor numérico de la CE individual de cada embrión (CE= Blast x Morfo) dividido por el total de embriones disponibles a día 3 de una paciente.

$$CETot = \sum Blast \times Morfo / N$$

CETot= media de la calidad del total de los embriones;
∑= sumatoria;
Blast= número de blastómeras ;
Morfo= grado de morfología embrionaria;
N= número de embriones al día 3.

b) la media del valor numérico de la calidad de los embriones transferidos (CETransf) fue calculada como la sumatoria de los valores numéricos de la calidad embrionaria (CE= Blast x Morfo) de los embriones transferidos dividido el total de embriones transferidos.

$$CETransf = \sum Blast \times Morfo / N$$

CETransf= media de la calidad de los embriones transferidos;
∑= sumatoria;
Blast= número de blastómeras;
Morfo= grado de morfología embrionaria
N= número de embriones transferidos al día 3

Evaluación de fragmentación de ADN en espermatozoides humanos

La fragmentación de ADN fue evaluada por el ensayo de TUNEL (del inglés **T**erminal **D**eoxynucleotidyl **T**ransferase–Mediated Deoxyuridine Triphosphate–Fluorescein Nick-End Labeling) utilizando el kit *In Situ Cell Death Detection*, *Fluorescein* (Roche Diagnostics GmbH, Mannheim, Germany); el ensayo utiliza desoxiuridina Trifosfato (dUTP) unido a fluoresceina para marcar simples y dobles roturas de cadenas de ADN. Se realizó de acuerdo a las especificaciones del fabricante y publicaciones anteriores (Barroso et al, 2000; Duran et al, 2002).

Cada suspensión de espermatozoides fue separada en gotas de 25 µL en un portaobjetos con 8 pocillos. Los espermatozoides fueron fijados durante 45 minutos a temperatura ambiente con 25 µL paraformaldehído al 4% (concentración final 2%). Se lavó con PBS/HSA para remover el fijador. Se permeabilizaron las células incubándolas durante 10 minutos a temperatura ambiente en una solución de TRITON X-100 al 0,1 % en 0,1 % de citrato de sodio. Se repitió el lavado con PBS/HSA y fue seguido por una incubación de 1 hora a 37ºC en la mezcla de reacción de TUNEL conteniendo deoxinucletidil transferasa terminal 0,5 UI/mL obtenida de timo de ternera, más dUTP unido a fluoresceína. El control negativo se realizó incubando una muestra con la misma mezcla de reacción pero sin el agregado de la enzima. El control positivo se realizó incubando una muestra con desoxirribonucleasa I (DNasa I) 1 U/mL por 20 minutos a temperatura ambiente previo a la incubación con la mezcla de reacción. Los controles fueron realizados en cada experimento (figura 8). Para proteger la fluorescencia, se agregó a cada muestra líquido de montar para fluorescencia (Vectashield; Vector Laboratories, Burlingame, CA, USA) previo a la evaluación. Fueron analizadas al azar 400 gametas (200 por pocillo) por muestra, utilizando un microscopio con objetivo de inmersión (1000 x) Nikon Eclipse E600 (Nikon, Tokio, Japon). Cada espermatozoide fue clasificado como ADN intacto (cuando no presentaba fluorescencia) o como ADN fragmentado (cuando mostraba una intensa fluorescencia verde en el núcleo).

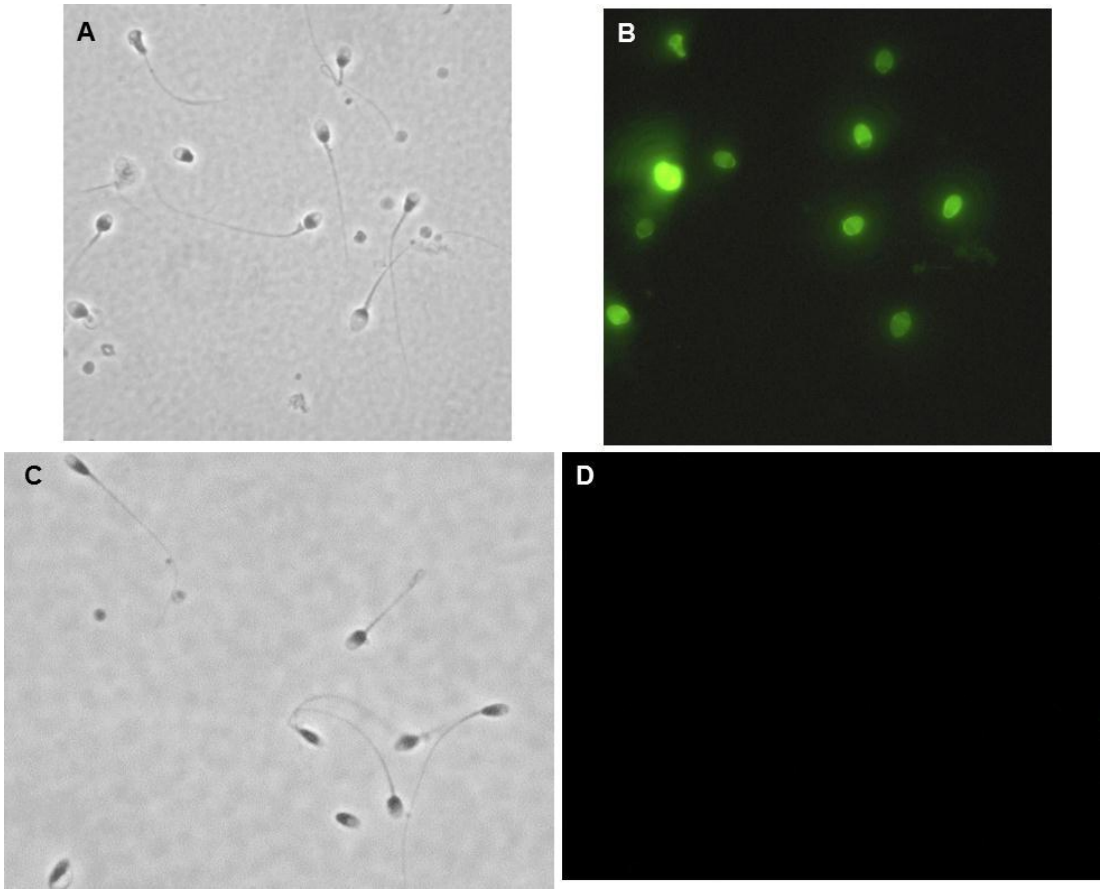


Figura 8. Fotomicrografía representativa de los controles positivo y negativo en la evaluación de la fragmentación de ADN *(lado izquierdo: contraste de fase; lado derecho: fluorescencia). A y B: control positvo, notar la fluorescencia (fragmentación de ADN) en todos los espermatozoides ; C y D: control negativo, notar la ausencia de fluorescencia en todos lo espermatozoides.*

Evaluación simultánea de la fragmentación de ADN y morfología espermática

Inmediatamente luego de la evaluación de la fragmentación de ADN y en la misma gota, se evaluó la morfología espermática (sin tinción) en diferentes campos elegidos al azar bajo microscopía de contraste de fase (Nikon Eclipse E600) equipada con una cámara SPOT-RT (Diagnostic Instruments, Inc., Sterling, MI) a 1000x bajo aceite de inmersión. Un total de 400 gametas fueron evaluadas en dos gotas por paciente. Durante el examen, cada vez que un espermatozoide normal era encontrado, el filtro fue inmediatamente cambiado para determinar la integridad del ADN. Los espermatozoides se consideraron normales cuando la cabeza tenía una forma normal, ovalada y simétrica, vacuolas ocupando menos del 20% del área de la cabeza, una

región acrosomal ocupando entre el 40% -70% de la zona de la cabeza, la cola inserta simétricamente y la ausencia de defectos en la pieza media o en la cola (WHO, 1999).

Evaluación de fragmentación de ADN en espermatozoides de ratón

Se utilizaron espermatozoides epididimarios de ratones Albino Swiss. La fragmentación de ADN fue evaluada por el ensayo de TUNEL. Se realizó de forma similar a la técnica utilizada para espermatozoides humanos (ver arriba). La única modificación fue el tiempo de exposición y temperatura de incubación de la DNasa I. Se incubó por 30 minutos y a 37°C. Todos los demás pasos fueron iguales a la técnica utilizada para espermatozoides humanos.

Evaluación de fragmentación de ADN en espermatozoides de ratón tratados con dieta enriquecida con ácidos grasos omega 3

Se analizó el impacto de la suplementación dietaria con omega 3 (ω3) sobre la integridad del ADN de los espermatozoides. Para ello, se administró aceite de hígado de bacalao conocido por contener altas cantidades de ácidos grasos poliinsaturados ω3: ácido ecosapentaenoico y docosahexaenoico. Dos grupos de animales adultos fueron alimentados *ad libitum* durante 3 meses con diferentes dietas: **Control** (alimento balanceado comercial; relación ω6/ω3 = 19; n=5) y **Problema** (enriquecida con aceite de hígado de bacalao: 95 g de alimento comercial con 5 g de aceite de hígado de bacalao; relación ω6/ω3 =1,1; n=5). Al finalizar el tratamiento, los animales fueron sacrificados mediante dislocación cervical y los espermatozoides fueron obtenidos mediante extrusión de los epidídimos en medio Tyrode. Inmediatamente, los gametos fueron fijados para la realización del ensayo de TUNEL.

Análisis estadístico

Los datos fueron analizados usando el software SPSS 14.0 (www.ibm.com). Los datos fueron expresados como la media ± la desviación estándar (X ± DE). Las comparaciones entre grupos fue realizada utilizando el test de Kruskal-Wallis. El test de Mann-Whitney fue usado para identificar diferencias significativas entre los grupos. El coeficiente de correlación Sperman fue usado para los análisis de correlación.

Los parámetros estudiados fueron la edad de ambos miembros de la pareja, la concentración, movilidad progresiva y morfología espermática, fragmentación de ADN en el total de espermatozoides y en la subpoblación de formas normales; calidad embrionaria (en los embriones totales y en los transferidos) y el embarazo clínico.

Las variables que fueron usadas para el análisis de correlación fueron edad del hombre y la mujer, los parámetros seminales, el número de ovocitos inseminados, el número de ovocitos fertilizados (diploides), las medias de la calidad embrionaria del total y de los embriones transferidos.

Para evaluar los factores determinantes de la calidad embrionaria tanto en la media de los embriones transferidos como en el total de embriones fertilizados, se realizaron análisis de regresión múltiple incluyendo los siguientes parámetros: edad del hombre y la mujer, el número de ovocitos inseminados, el número de ovocitos fertilizados, morfología de los espermatozoides (por contraste de fase), la fragmentación de ADN en el total de los espermatozoides y en la subpoblación de espermatozoides normales después de la técnica de *swim-up*.

Además, para evaluar la capacidad de predecir embarazo del porcentaje de espermatozoides normales con fragmentación de ADN y la calidad embrionaria de los embriones transferidos fueron construidas curvas ROC. Un análisis de regresión logística se utilizó para evaluar el valor de los parámetros seleccionados para predecir embarazo mediante los cocientes de probabilidad. Los parámetros evaluados fueron: edad de los hombres y mujeres, el número de ovocitos inseminados y fertilizados, morfología de los espermatozoides (contraste de fase) post-*swim-up*, la fragmentación de ADN en el total y en la subpoblación de formas normales de espermatozoides y la media de la calidad embrionaria en el total de los embriones fertilizados y en los transferidos.

Valores de $p \leq 0{,}05$ fueron considerados estadísticamente significativos.

RESULTADOS

RESULTADOS

Estudio de la fragmentación de ADN y su impacto en ICSI

La primera etapa de esta tesis fue estudiar la posible relación de la presencia de espermatozoides con ADN fragmentado y su impacto en los resultados de ICSI. Para ello se analizó una fracción de la misma muestra de espermatozoides de 82 hombres integrantes de parejas sometidas a ICSI.

Características seminales de las muestras evaluadas

La media del volumen seminal fue de 3,2 ± 1,5 mL (rango entre 0,2 mL y 6,5 mL); la concentración fue de 80,9 ± 69,8 x 10^6/mL (rango entre 1,5 x 10^6 / mL y 404 x 10^6 / mL); la movilidad fue de 54,7 ± 18,5% (rango entre 11 % y 95,1 %) y la morfología fue de 5,4 ± 4.9 % (rango entre 0 % y 16 %).

Solo el 5 % (4 de 82 muestras) de las muestras no presentaron alteraciones en los parámetros seminales evaluados (normozoospermia). La anomalía predominante fue la teratozoospermia. El 93 % (76 de 82 muestras) de la muestras evaluadas presentaba anomalías en la cabeza (teratozoospermia). El 48% (39 de 82 muestras) mostraba alteraciones únicamente en la morfología, mientras que 27 % (22 de 82 muestras) lo hacía en conjunto con la movilidad (astenoteratozoospermia), el 11% (9 de 82 muestras) en conjunto con la concentración (oligoteratozoospermia) y el 7 % (6 de 82) presentaba perturbaciones en los tres parámetros (ologoastenoteratozoospermia). El 1 % presentó solamente astenozoospermia y otro 1 % presentó solamente hipospermia.

Evaluación de fragmentación de ADN en pacientes de ICSI

Se realizó el ensayo de TUNEL a cada muestra. Los patrones observados fueron: fluorescencia en la cabeza del espermatozoide (TUNEL positivo= ADN fragmentado) y ausencia de fluorescencia en la cabeza del espermatozoide (TUNEL negativo= ADN intacto). Los datos fueron luego comparados con los resultados del procedimiento.

El 37,8% (31 de 82 pacientes) de los pacientes lograron un embarazo confirmado por βHCG y visualización por ecografía de saco gestacional a las 7 semanas. No hubo diferencias significativas en los protocolos de hiperestimulación ovárica entre las pacientes que lograron el embarazo y las que no lo lograron. No hubo diferencias estadísticamente significativas, tanto en la edad de las mujeres, en la edad de los hombres, el número de ovocitos recuperados y el número de ovocitos inseminados entre el grupo de mujeres que lograron el embarazo y las que no lo lograron. Los resultados fueron se consignan en la tabla 2.

Tabla 2. Valores de los parámetros analizados en los pacientes que embarazaron y los que no lo lograron.

Grupo	Embarazo (n=15)	No Embarazo (n=21)
Edad de la mujer	34.3 ± 4.5	34.2 ± 4.4
Edad del hombre	36.5 ± 5.2	36.4 ± 4.2
Ovocitos recuperados	9.9 ± 4.5	11.3 ± 5.1
Tasa de Fertilización (%)	83 ± 16	73 ± 20
Embriones transferidos	2.4 ± 0.5	2.3 ± 0.5
Concentración espermática seminal (x 10^6 / mL)	73.6 ± 55.7	75.1 ± 65.7
Movilidad espermática seminal (%)	49.6 ± 17.7	55.4 ± 20.9
Morfología espermática seminal (%)	5.2 ± 4.8	5.5 ± 5.5
Fragmentación de ADN en el total de espermatozoides	15.6 ± 8.5	13.3 ± 12.3
Fragmentación de ADN en espermatozoides normales	**18.9 ± 20.0[a]**	**33.8 ± 19.4[a]**
[a] Diferencia significativa (p = 0,03)		

Los valores fueron expresados como la media (M) ± la desviación estándar (DE).

Con respecto a los resultados de ICSI, no hubo diferencias significativas entre ambos grupos en la tasa de fertilización, así como en la calidad embrionaria del total de los embriones inseminados. Como era de esperar, la calidad de los embriones transferidos fue mayor en las mujeres que lograron el embarazo en comparación con las

que no lo lograron (p=0,04; tabla 2), mientras que en ambos grupos se observaron cifras semejantes en el porcentaje de espermatozoides con ADN fragmentado.

Por otra parte, cuando se aplicó el coeficiente de *Spearman* no se detectó correlación entre el porcentaje de espermatozoides fragmentados y la tasa de fertilización (r=0,059; p=0,597) o la media de la calidad del total de los embriones (r=-0,096; p= 0,392) (figura 9).

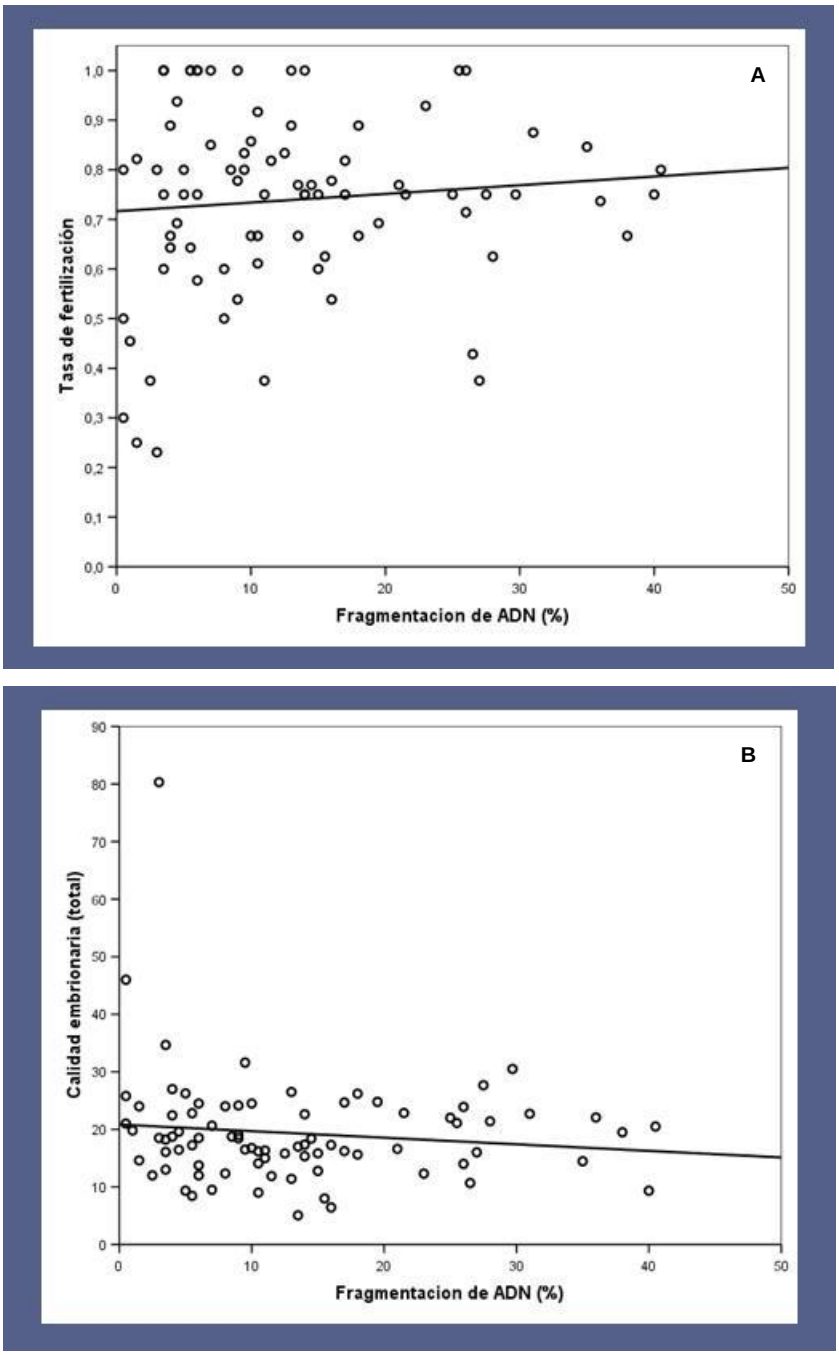


Figura 9. Correlaciones entre fragmentación de ADN y calidad embrionaria*. (A) Gráficos de dispersión y de regresión lineal entre el porcentaje de espermatozoides con fragmentación de ADN y la tasa de fertilización, r=0,059; p=0,597. (B) Gráficos de dispersión y de regresión lineal entre el porcentaje de espermatozoides con fragmentación de ADN y la media de la calidad embrionaria de los embriones totales, r=-0,096; p= 0,392.*

Parámetros seminales y fragmentación de ADN

El análisis estadístico (coeficiente de *Spearman*) no detectó en estas muestras correlación entre los parámetros seminales y el porcentaje de fragmentación de ADN. Los resultados están expuestos en la tabla 3.

Tabla 3. Correlación y significación estadística entre los parámetros seminales y la fragmentación de ADN

		Fragmentación de ADN
Volumen	Coeficiente de Correlación	0,022
	Significación	0,84
Concentración	Coeficiente de Correlación	-0,044
	Significación	0,69
Movilidad	Coeficiente de Correlación	-0,058
	Significación	0,60
Morfología	Coeficiente de Correlación	-0,015
	Significación	0,89

Estudio de la fragmentación de ADN en subpoblaciones de espermatozoides con diferentes morfologías

Para conocer la presencia y distribución de la fragmentación de ADN en los espermatozoides con diferentes patrones de morfología, se evaluó en forma simultánea y en la misma célula, fragmentación de ADN y morfología espermática como se describe en materiales y métodos. Se analizaron 7595 células con fragmentación de ADN de 24 muestras de pacientes infértiles con más del 10% de fragmentación de ADN en pacientes infértiles.

La anomalía morfológica predominante en espermatozoides con ADN fragmentado fue de amorfos (45,3%), seguida por los espermatozoides vacuolados. Solo

el 1,9 % de los espermatozoides con ADN fragmentado mostraban una morfología característica de espermatozoides considerados morfológicamente normales (Figura 10). Imágenes representativas de cada morfología son mostradas en la figura 11.

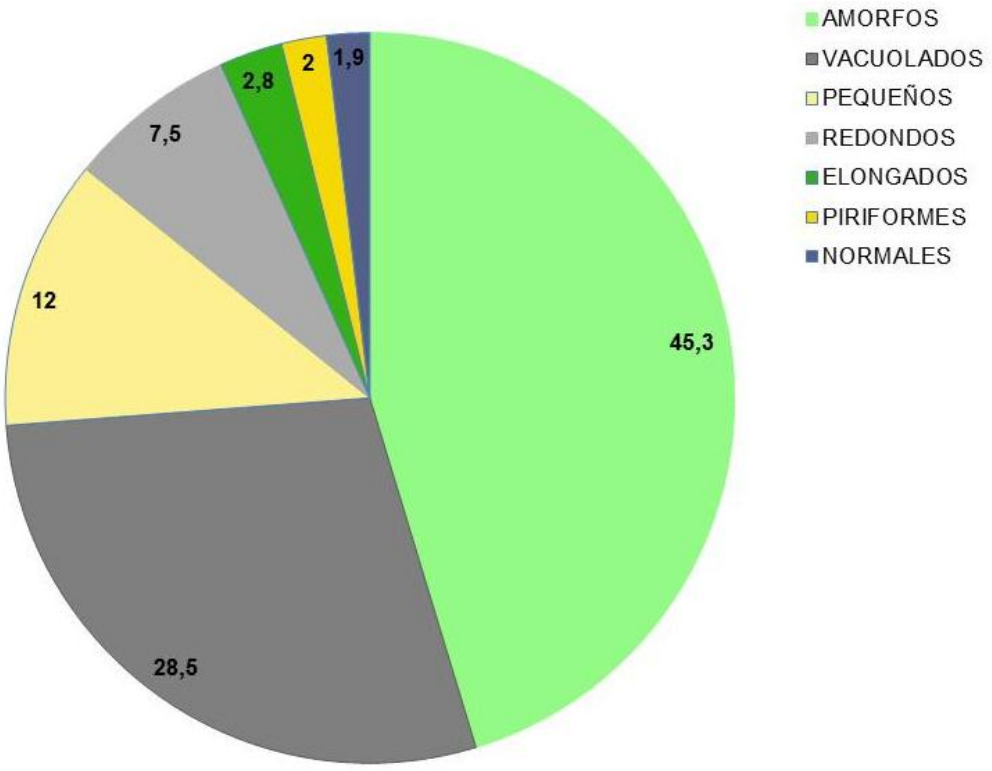


Figura 10. Distribución porcentual de variantes morfológicas en espermatozoides con ADN fragmentado (n=7595).

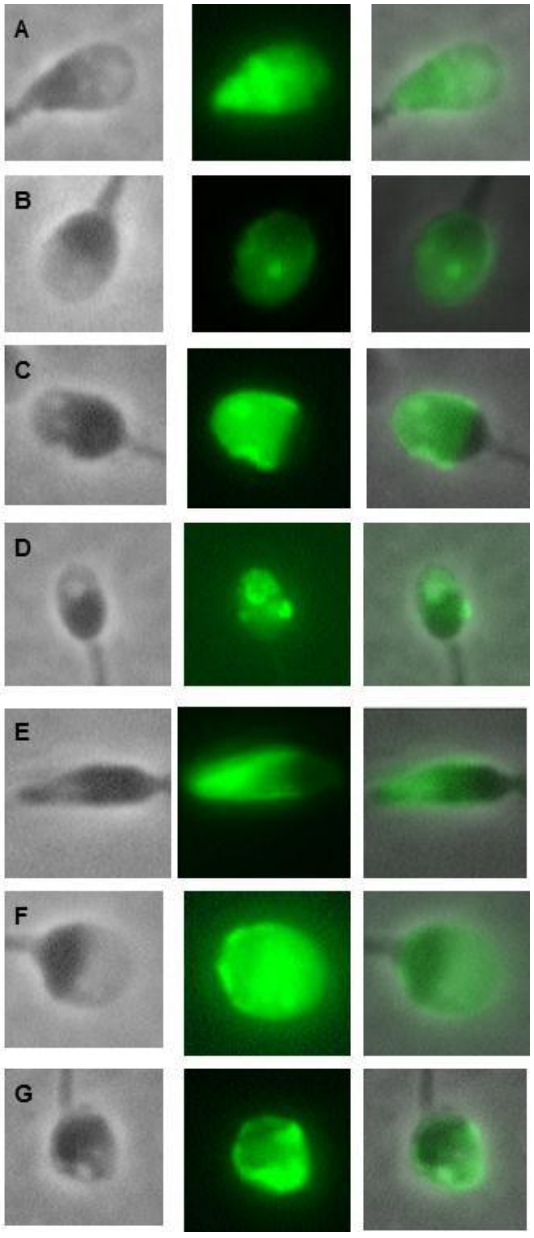


Figura 11. Fotomicrografia representativa de la evaluación simultánea de morfología espermática *(contraste de fase: columna izquierda), fragmentación de ADN (fluorescencia: columna del centro) y la unión de las dos primeras (contraste de fase y fluorescencia: columna derecha). Linea A: Piriformes; Linea B: Normal; Linea C: Vacuolados; Linea D: Pequeños; Linea E: elongados; Linea F: Redondos y Linea G: Amorfos.*

Por otro lado, cuando se evaluó la subpoblación de espermatozoides morfológicamente normales, el 31 % de las gametas presentaba fragmentación de ADN (figura 12).

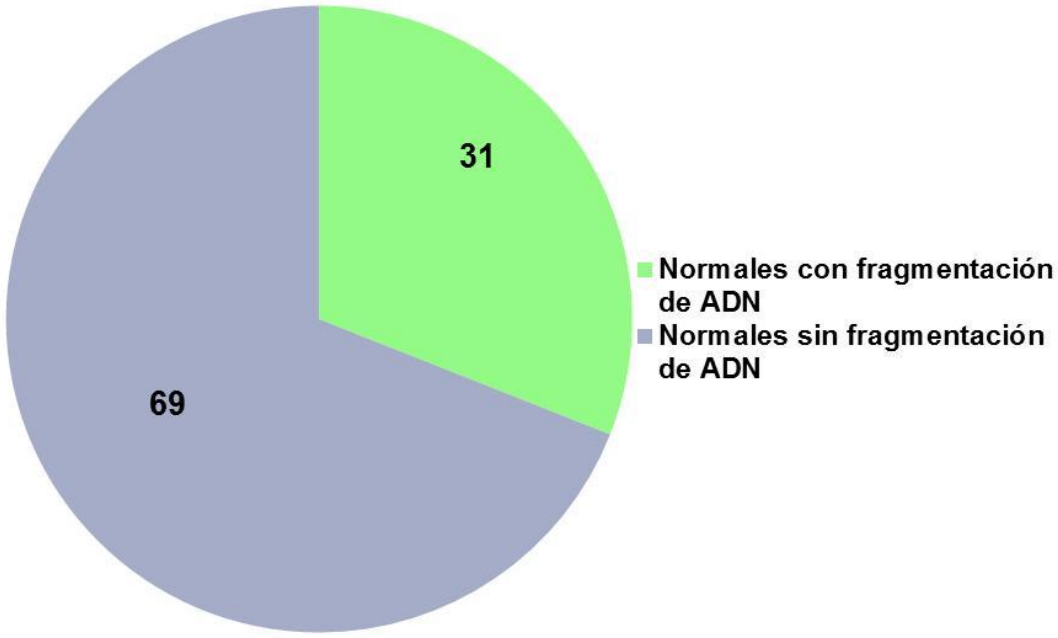


Figura 12. Distribución porcentual de espermatozoides morfológicamente normales, con o sin fragmentación de ADN*(n=7595).*

Fragmentación de ADN en espermatozoides morfológicamente normales

Los espermatozoides morfológicamente normales son las gametas que fertilizarán al ovocito en el momento de la concepción. Por lo tanto, nos propusimos determinar la incidencia de la fragmentación de ADN espermático en espermatozoides con aspecto morfológico normal que fueron obtenidas de la fracción del *Swim-Up* con espermatozoides altamente móviles. Se estudiaron muestras de hombres fértiles (FER), subfértiles (SF) e infértiles (INF). La tabla 4 muestra las características seminales (concentración, movilidad progresiva y morfología) de los grupos analizados. Las anormalidades morfológicas en todos los casos fueron predominantemente en la cabeza

de los espermatozoides (principalmente cabezas severamente amorfas), con presencia ocasional de vacuolas y defectos en la pieza media (gota citoplasmática); menos del 5 % de los espermatozoides estudiados presentaron defectos en la cola. No hubo diferencias estadísticamente significativas en la concentración espermática ($p > 0,05$) ni en la movilidad progresiva ($p > 0,05$) entre los grupos estudiados. La morfología espermática normal en el grupo FER fue significativamente más alta ($p< 0,05$) en comparación de los grupos SF e INF. No hubo diferencias significativas entre los grupos SF e INF (tabla 4).

Tabla 4. Concentración, movilidad y morfología espermática en las muestras de semen de hombres fértiles (FER), subfértiles (SF) e infértiles (INF) estudiados.

Paciente	Concentración (10^6 / mL) [a]	Movilidad (%) [b]	Morfología Normal (%) [c]
FER 1	29,0	56,0	10,5
FER 2	23,4	66,0	12,0
FER 3	55,1	65,0	11,0
FER 4	60,3	70,0	11,0
SF1	56,0	51,0	4,0
SF2	37,0	59,0	3,5
SF3	99,0	77,0	7,5
SF4	44,5	75,0	3,0
SF5	26,0	24,0	3,5
INF1	82,0	95,1	3,5
INF2	71,0	63,4	5,0
INF3	58,0	41,4	2,0
INF4	136,0	60,0	7,0
INF5	97,5	59,0	4,5
INF6	4,0	63,0	1,5
INF7	32,0	47,0	3,0
INF8	201,5	52,0	3,0
INF9	174,5	49,0	4,0
INF10	64,0	84,4	5,0

[a] $p > 0,05$ comparando concentración en los tres grupos.
[b] $p > 0,05$ comparando movilidad en los tres grupos.
[c] $p < 0,05$ comparando FER versus SF y FER versus INF.

Por otra parte, en los 3 grupos considerados se seleccionaron espermatozoides móviles mediante la técnica de *Swim-Up* (ver materiales y métodos) y se realizó en ensayo de TUNEL mediante microscopía de fluorescencia. Luego se procedió a la evaluación simultánea de morfología espermática y fragmentación de ADN por microscopía de contraste de fase y fluorescencia. La proporción de células con TUNEL positivo fue de 3,9 % ± 2,9 % para el grupo FER, 9,8 % ± 5,5 % para el grupo SF y 21,2 % ± 13,4 % para el grupo INF. Se observó una diferencia estadísticamente significativa entre los grupos FER e INF ($p < 0,05$). Los resultados se exponen en la Figura 13.

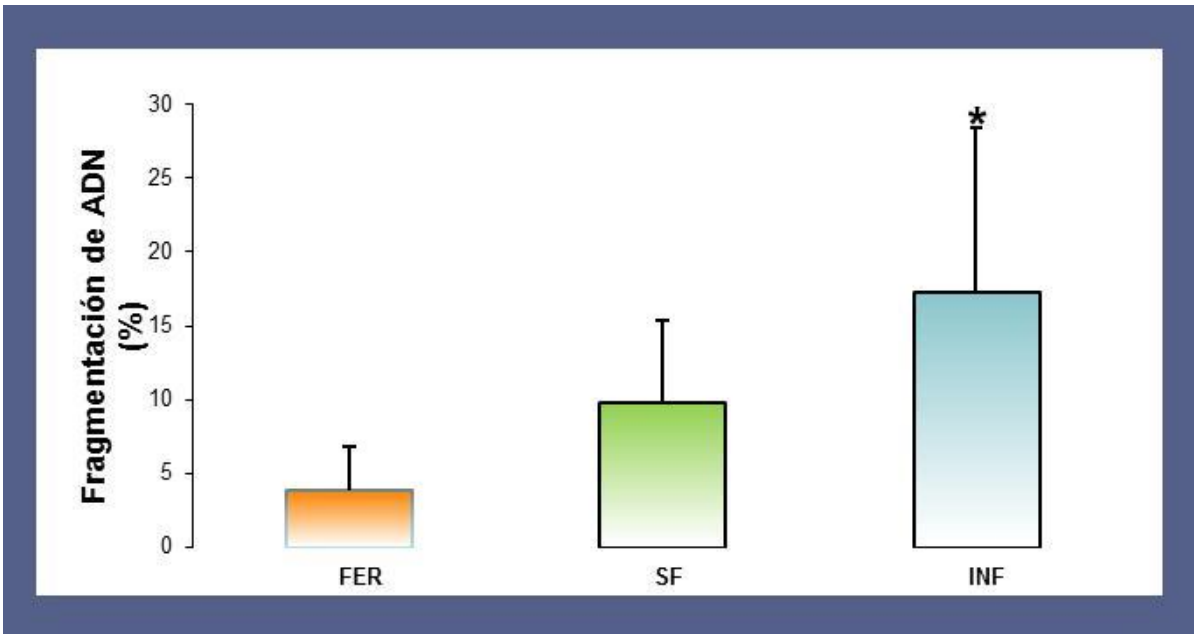


Figura 13. Incidencia de espermatozoides con fragmentación de ADN *(TUNEL positivo) en grupos de hombres fértiles (FER; n=4), subfértiles (SF;n=5) e infértiles (INF;n=10). Los resultados se expresan como media; las líneas verticales representan la desviación estandar. ∗ FER vs INF: p< 0,05.*

El porcentaje de espermatozoides morfológicamente normales, examinados por microscopía por contraste de fase fue el siguiente: grupo FER 7,5 % ± 0,6 % (n=4), el grupo SF 1,3 % ± 1,7 %(n=5) y el grupo INF 1,0 % ± 0,3 % (n=10) ($p< 0,05$, FER vs INF). Seguidamente, los espermatozoides con morfología normal fueron examinados por microscopia de fluorescencia mediante el ensayo de TUNEL.

Las figuras 14 y 15 muestran fotomicrografías representativas de lo observado. No se encontró fragmentación de ADN en los espermatozoides morfológicamente normales de las muestras del grupo FER. En una sola muestra del grupo SF se observó espermatozoides con formas normales exhibiendo fluorescencia (TUNEL positivo). Sin embargo, en todas las muestras estudiadas de pacientes infértiles (grupo INF) se encontraron espermatozoides morfológicamente normales con fragmentación de ADN ($p< 0,05$ en INF vs FER). Los resultados fueron resumidos en la TABLA 5.

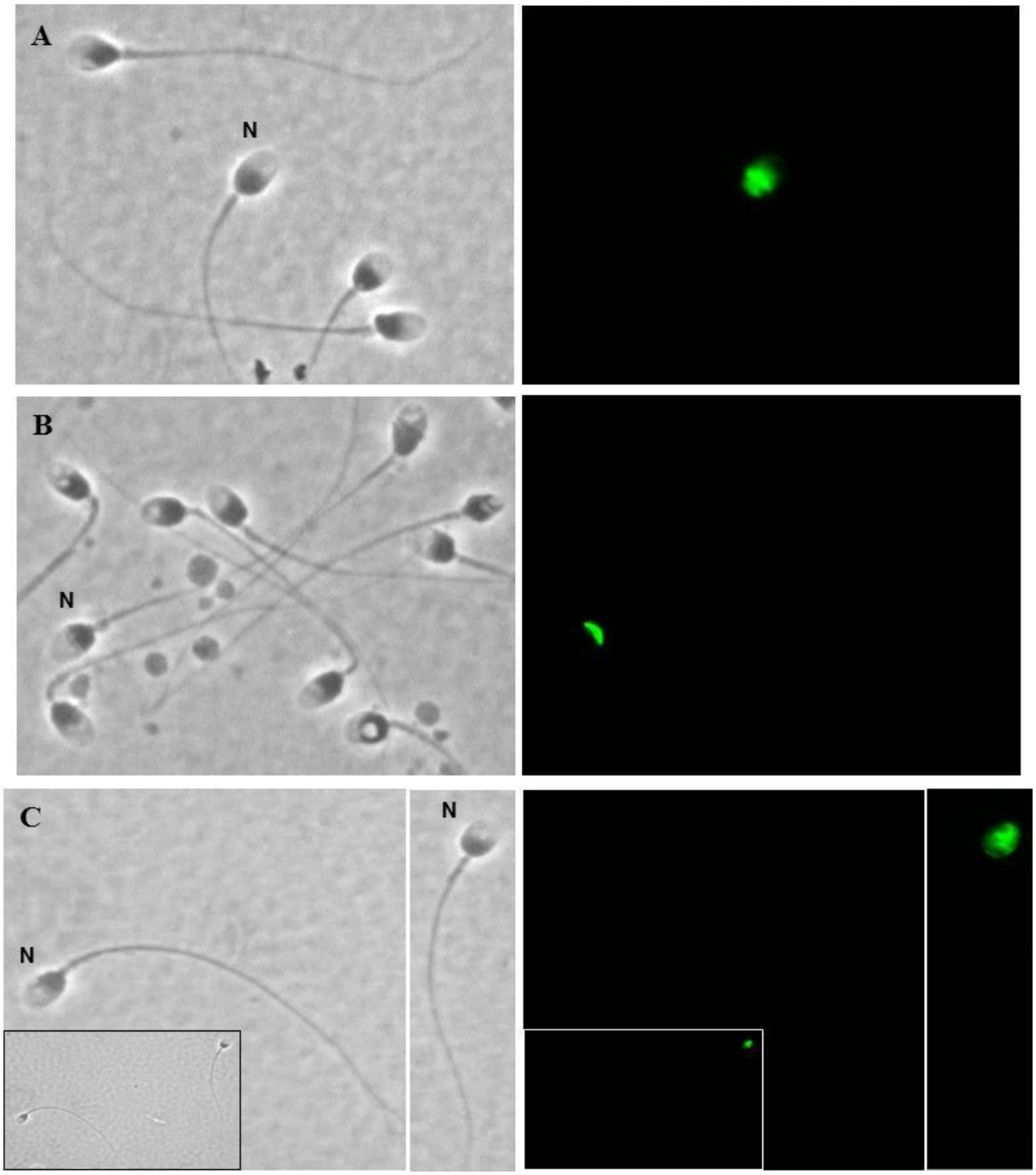


Figura 14. Ejemplos de imágenes típicas obtenidas en la evaluación simultánea de espermatozoides *luego de la separación por Swim- up con morfología normal y fragmentación de ADN (lado izquierdo: contraste de fase; lado derecho: fluorescencia). A: un espermatozoide normal (N) con fragmentación de ADN, notar en la misma imagen tres espermatozoides con diferentes grados de morfología anormal sin fragmentación de ADN; B: un espermatozoide normal (N) con fragmentación de ADN, notar una gran cantidad de espermatozoides amorfos sin fragmentación de ADN; C: dos espermatozoides normales (N) uno de ellos con fragmentación de ADN.*

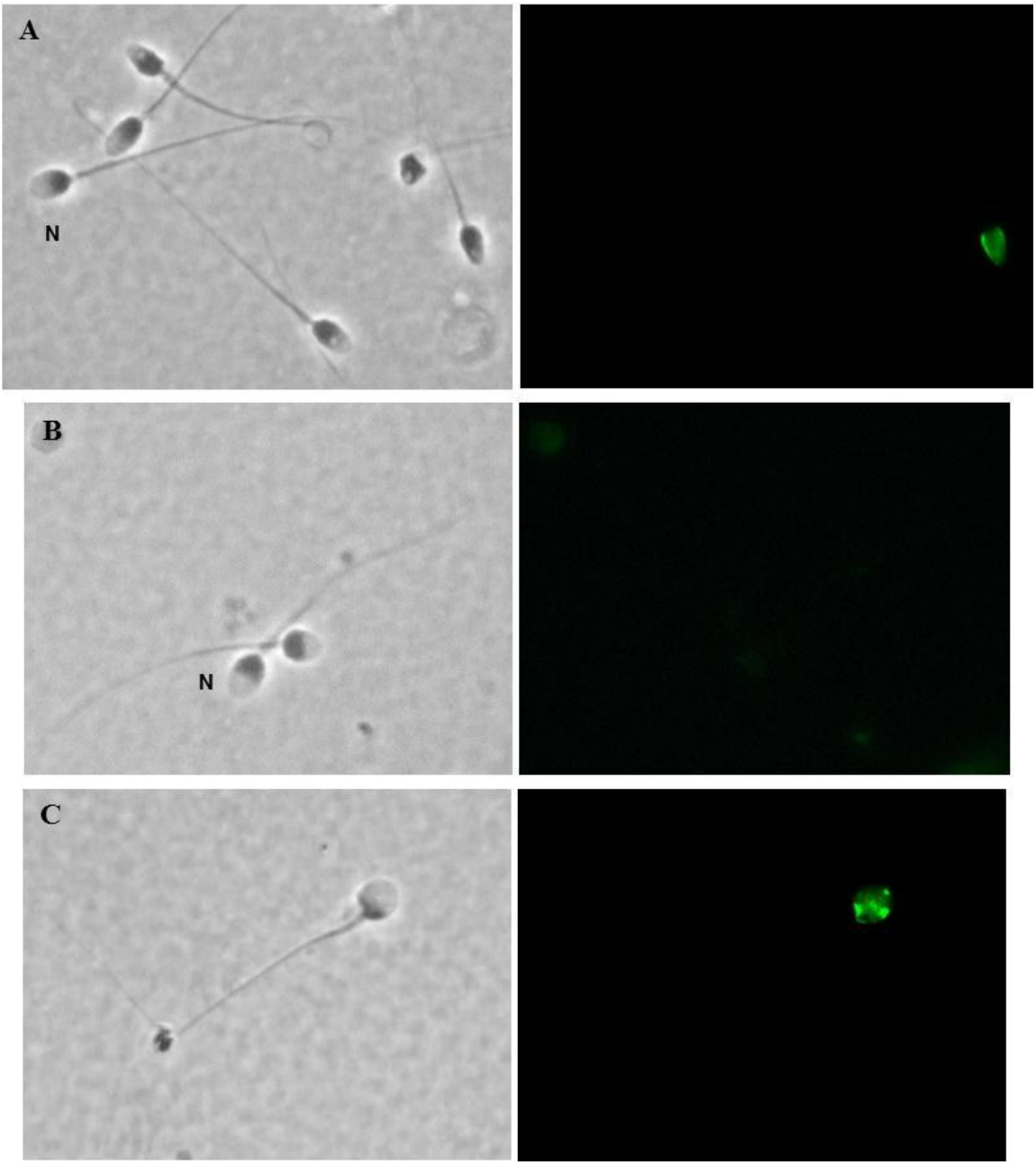


Figura 15. Ejemplos de imágenes obtenidas en la evaluación simultánea de espermatozoides luego de la separación por Swim-up de morfología y fragmentación de ADN *(lado izquierdo: contraste de fase; lado derecho: fluorescencia). A: un espermatozoide normal (N) sin fragmentación de ADN; notar varios espermatozoides anormales, uno de ellos con fragmentación de ADN; B: un espermatozoide normal (N) y un espermatozoide anormal sin fragmentación de ADN; C: un espermatozoide anormal con fragmentación de ADN.*

Tabla 5. Espermatozoides seleccionados por *Swim-Up*: porcentaje de espermatozoides con fragmentación de ADN, porcentaje de espermatozoides con morfología normal y porcentaje de espermatozoides normales con fragmentación de ADN.

Paciente	Fragmentación de ADN(%) [a]	Espermatozoides con mofología normal (%) [b]	Espermatozoides normales con fragmentación de ADN (%) [c]
FER 1	0.5	7.00	**0.0 (0/28)**
FER 2	6.5	8.00	**0.0 (0/32)**
FER 3	6.0	8.00	**0.0 (0/32)**
FER 4	2.5	7.00	**0.0 (0/28)**
SF1	4.0	1.00	**0.0 (0/4)**
SF2	5.0	1.25	**0.0 (0/5)**
SF3	12.5	4.25	**47.0 (8/17)**
SF4	17.5	0.25	**0.0 (0/1)**
SF5	10.0	0.00	**0.0 (0/0)**
INF1	5.0	1.00	**50 (2/4)**
INF2	23.0	1.25	**60.0 (3/5)**
INF3	5.5	0.75	**33.3 (1/3)**
INF4	27.0	1.50	**50.0 (3/6)**
INF5	26.0	0.75	**66.6 (2/3)**
INF6	38,51	0,5	**50 (1/2)**
INF7	39,5	1,75	**42,9 (3/7)**
INF8	31	1	**25 (1/4)**
INF9	10,5	1,25	**40 (2/5)**
INF10	6,5	1,25	**20 (1/5)**

Resultados obtenidos de las muestras individuales de Swim-Up de los tres grupos de hombres estudiados; FER: fértiles; SF: subfértiles; INF: infértiles. Los resultados son expresados como: % de Fragmentación de ADN (número de espermatozoides TUNEL positivo / 200 espermatozoides), % espermatozoides con morfología normal (número de espermatozoides normales / 400 espermatozoides) y la proporción de espermatozoides con morfología normal que presentaron TUNEL positivo, calculados como el número de espermatozoides morfológicamente normales con TUNEL positivo / el número total de espermatozoides con morfología normal contados. [a] $p < 0,05$ *FER versus INF.* [b] $p < 0,05$ *FER versus SF y FER versus INF.* [c] $p < 0,05$ *FER versus SF y FER versus INF.*

Fragmentación de ADN en espermatozoides morfológicamente normales y su impacto en ICSI

Los espermatozoides móviles y morfológicamente normales son las células, entre las cuales una será seleccionada e inyectada dentro del ovocito para la realización del ICSI.

Luego de comprobar que en pacientes infértiles los espermatozoides morfológicamente normales podrían tener el ADN fragmentado, se evaluó el impacto de los espermatozoides de apariencia normal con ADN dañado sobre los resultados de ICSI. Se compararon parejas que lograron el embarazo luego de la transferencia embrionaria (n= 15) versus las parejas que no lograron el embarazo (n = 21). La tabla 6 describe el número de muestras clasificadas en diferentes grupos de acuerdo al análisis de semen. Una alta proporción de muestras presentaba solo teratozoospermia (moderada o severa), mientras que un pequeño porcentaje de individuos tenía baja concentración y/o movilidad espermática, con o sin coexistencia de teratozoospermia.

Tabla 6. Clasificación de las muestras estudiadas (n= 36) de acuerdo a los parámetros seminales básicos

Análisis de semen	N	%
Oligozoospermia aislada (< 20 x 10^6 / mL)	1	2,8
Astenozoospermia aislada (< 50 % movilidad progresiva)	1	2,8
Teratozoospermia aislada		
Moderada (5% a 9% de formas normales)	15	41,7
Severa (≤ 4% de formas normales)	10	27,7
OligoTeratozoospermia	3	8,3
AstenoTeratozoospermia	4	11,1
OligoAstenoTeratozoospermia	2	5,6

Tal como se muestra en tabla 7 no se observaron diferencias estadísticamente significativas en el número de ovocitos recuperados, ni en la tasa de fertilización o en el número de embriones transferidos entre los dos grupos.

Similar a nuestros previos resultados la edad de las mujeres, la edad de los hombres, los parámetros seminales básicos o el porcentaje de fragmentación de ADN en el total de espermatozoides no mostró diferencias significativas cuando se comparó el grupo de pacientes que lograron el embarazo con los que no lo lograron. Contrariamente, el porcentaje de fragmentación de ADN en los espermatozoides

morfológicamente normales fue estadísticamente mayor en el grupo de los que no lograron el embarazo.

Tabla 7. Comparación de la edad de la mujer y el hombre, número de ovocitos recuperados, tasa de fertilización, embriones transferidos, parámetros seminales, fragmentación de ADN en el total de los espermatozoides y fragmentación de ADN en los espermatozoides morfológicamente normales.

Grupo	Embarazo (n=15)	No Embarazo (n=21)
Edad de la mujer	34.3 ± 4.5	34.2 ± 4.4
Edad del hombre	36.5 ± 5.2	36.4 ± 4.2
Ovocitos recuperados	9.9 ± 4.5	11.3 ± 5.1
Tasa de Fertilización (%)	83 ± 16	73 ± 20
Embriones transferidos	2.4 ± 0.5	2.3 ± 0.5
Concentración espermática seminal (x 10^6 / mL)	73.6 ± 55.7	75.1 ± 65.7
Movilidad espermática seminal (%)	49.6 ± 17.7	55.4 ± 20.9
Morfología espermática seminal (%)	5.2 ± 4.8	5.5 ± 5.5
Fragmentación de ADN en el total de espermatozoides	15.6 ± 8.5	13.3 ± 12.3
Fragmentación de ADN en espermatozoides normales	**18.9 ± 20.0[a]**	**33.8 ± 19.4[a]**
[a] Diferencia significativa (p = 0,03)		

Asimismo, no hubo correlación entre el porcentaje de espermatozoides morfológicamente normales con ADN fragmentado y el porcentaje de espermatozoides morfológicamente normales, ni con la movilidad y concentración espermática. Por otro lado, no hubo correlación entre el porcentaje de espermatozoides morfológicamente normales con fragmentación de ADN y el porcentaje de fragmentación de ADN en el total de espermatozoides (figura 16).

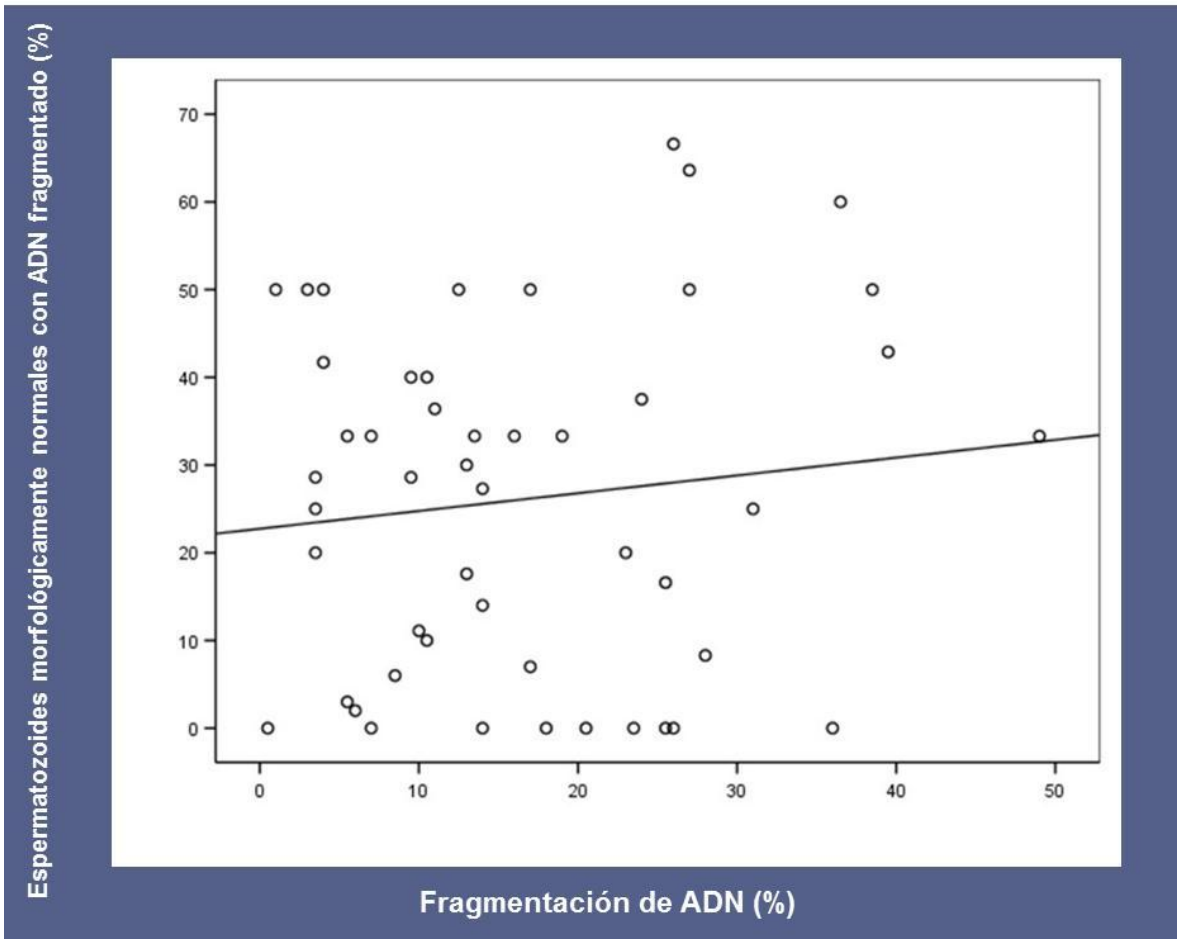


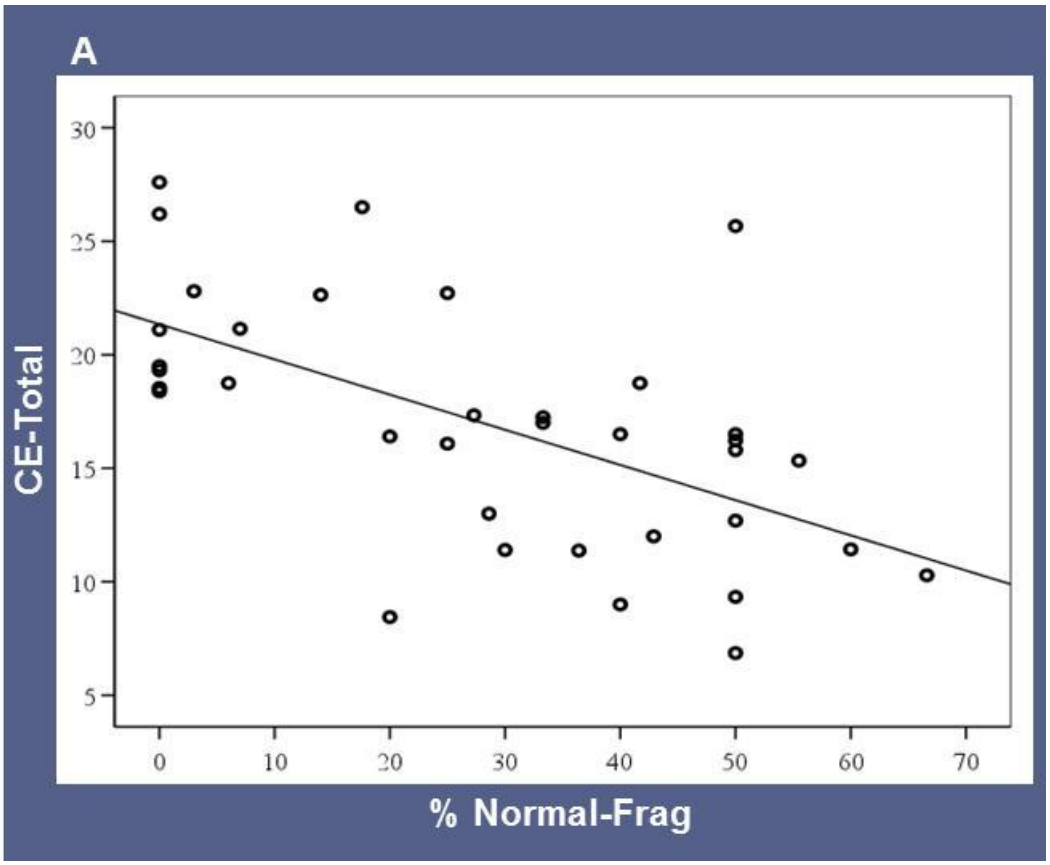
Figura 16. ***Correlación entre el porcentaje de espermatozoides con morfología normal con fragmentación de ADN y el porcentaje de la fragmentación de ADN en el total de espermatozoides evaluados***. *r=-0,048, p = 0745.*

Determinantes de la calidad embrionaria

La calidad embrionaria es un factor importante en el éxito de un tratamiento de fertilización *in vitro*. Todos los parámetros evaluados fueron utilizados para determinar si guardaban alguna correlación con la calidad de los embriones.

Los análisis no paramétricos demostraron una correlación negativa, estadísticamente significativa, entre el porcentaje de espermatozoides normales con fragmentación de ADN y la media de la calidad embrionaria del total de embriones (r= -0,64, p< 0,001) así como entre el porcentaje de espermatozoides normales fragmentados y la media de la calidad embrionaria de los embriones transferidos (r= -0,63, p< 0,001)

(Figura 17). El análisis de regresión múltiple (r= 0,78, SE+ 3,9 df= 29, p < 0,001) demostró que el porcentaje de espermatozoides normales con fragmentación de ADN (B= -0,19, p< 0,001) y la edad de la mujer (B= -0,38, p=0,03) fueron los principales determinantes la calidad embrionaria de los embriones transferidos. Asimismo, el porcentaje de espermatozoides normales con fragmentación de ADN (B= -018, p< 0,001) y el número de ovocitos fertilizados (B= -0,44, p < 0,03) fueron los principales determinantes en la calidad embrionaria del total de embriones evaluados por análisis de regresión múltiple (r=0,73, SE=3,9, df=29, p<0,001).



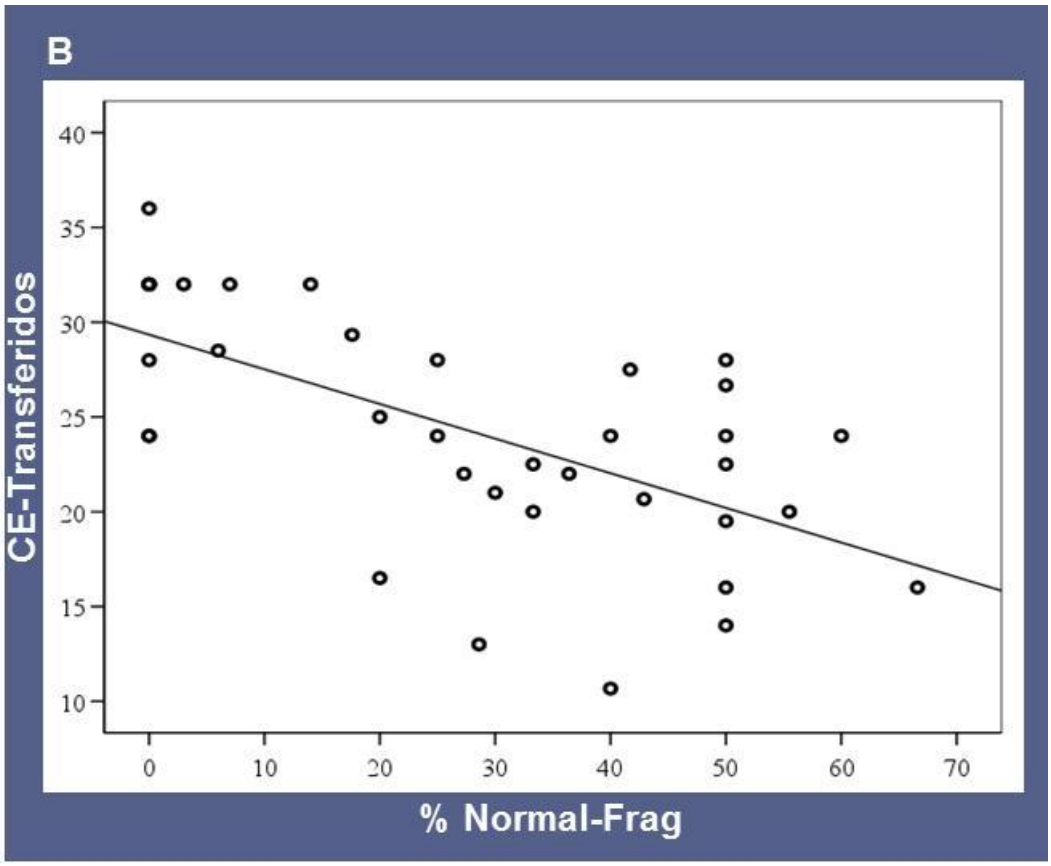


Figura 17. Correlaciones entre el porcentaje de espermatozoides con morfología normal con fragmentación de ADN y la calidad embrionaria. *(A) Gráficos de dispersión y de regresión lineal entre el porcentaje de espermatozoides normales con fragmentación de ADN (%Normal-Frag) y la media de la calidad embrionaria del total de los embriones (CE-Total), r=-0,64, p < 0,001. (B) Gráficos de dispersión y de regresión lineal entre el porcentaje de espermatozoides normales con fragmentación de ADN (Normal-Frag) y la media de la calidad embrionaria de los embriones transferidos (CE-Transferidos), r= -0,63, p < 0,001.*

Predictores de embarazo

El análisis de curvas ROC demostró que el porcentaje de espermatozoides normales con fragmentación de ADN y la media de la calidad embrionaria de los

embriones transferidos es un predictor útil de la probabilidad de embarazo (figura 18). La tabla 8 resume los resultados del análisis de curvas ROC. El área bajo la curva (0,7 y 0,75), los valores p (<0,021 y < 0,002) y la especificidad (82,6 y 91,3) para el porcentaje de espermatozoides normales con fragmentación de ADN y la media de la calidad embrionaria de los embriones transferidos respectivamente, mostró un poder predictivo de embarazo estadísticamente significativo.

Usando el análisis de curvas ROC también se determinó el valor óptimo de corte para la predicción de los resultados de embarazo. De acuerdo a estos resultados, cuando la media de la calidad embrionaria de los embriones transferidos fue mayor de 28 había un incremento de 6,2 veces en la probabilidad de lograr un embarazo, pero cuando la media de la calidad embrionaria de los embriones transferidos era menor o igual a 28, había una disminución de 0,5 veces en la probabilidad de embarazo.

Asimismo, si el porcentaje de espermatozoides normales con fragmentación de ADN era menor o igual a 17,6 % la probabilidad de lograr un embarazo era 3,5 veces mayor, pero si el porcentaje de espermatozoides normales con fragmentación de ADN era mayor a 17,6 % el potencial de embarazo disminuía a la mitad (tabla 8). Un análisis de regresión logístico estimó un modelo para predecir el embarazo (-2 log de la probabilidad= 33,124, prueba X^2= 6,305 con un grado de libertad, p= 0,012).

Tabla 8. Resumen del análisis de la curva ROC: capacidad para predecir embarazo del porcentaje de espermatozoides normales con fragmentación de ADN y la media de la calidad embrionaria de los embriones transferidos.

Parámetro	Área bajo la curva	95% intervalo de confianza	P	Punto de corte	Sensibilidad	Sensibilidad	Razón de probabilidad positiva	Razón de probabilidad negativa	Valor predictivo positivo	Valor predictivo negativo
Fragmentación de ADN en espermatozoides normales (%)	0.70	0.53-0.84	**0.02**	**≤17.6%**	61.5	82.6	3.5	0.5	66.7	79.2
Calidad embrionaria de gametas transferidas	0.75	0.58-0.88	**0.005**	**>28**	53.9	91.3	6.2	0.5	77.8	77.8

Solo la media de la calidad de los embriones transferidos fue incluido en este modelo (Exp B= 1,1211, p=0,03), probablemente porque este fue el único mejor predictor independiente de la concepción. Cuando el análisis de regresión logístico fue realizada excluyendo de la lista de los parámetros evaluados la calidad embrionaria de los embriones transferidos, el modelo estimado para predecir embarazo incluyó solamente al porcentaje de espermatozoides normales con fragmentación de ADN (Exp B= 0,958, p= 0,04) entre todos los parámetros evaluados (-2log de probabilidad= 34,321, prueba X^2= 5,109 con un grado de libertad, p= 0,024; figura 18).

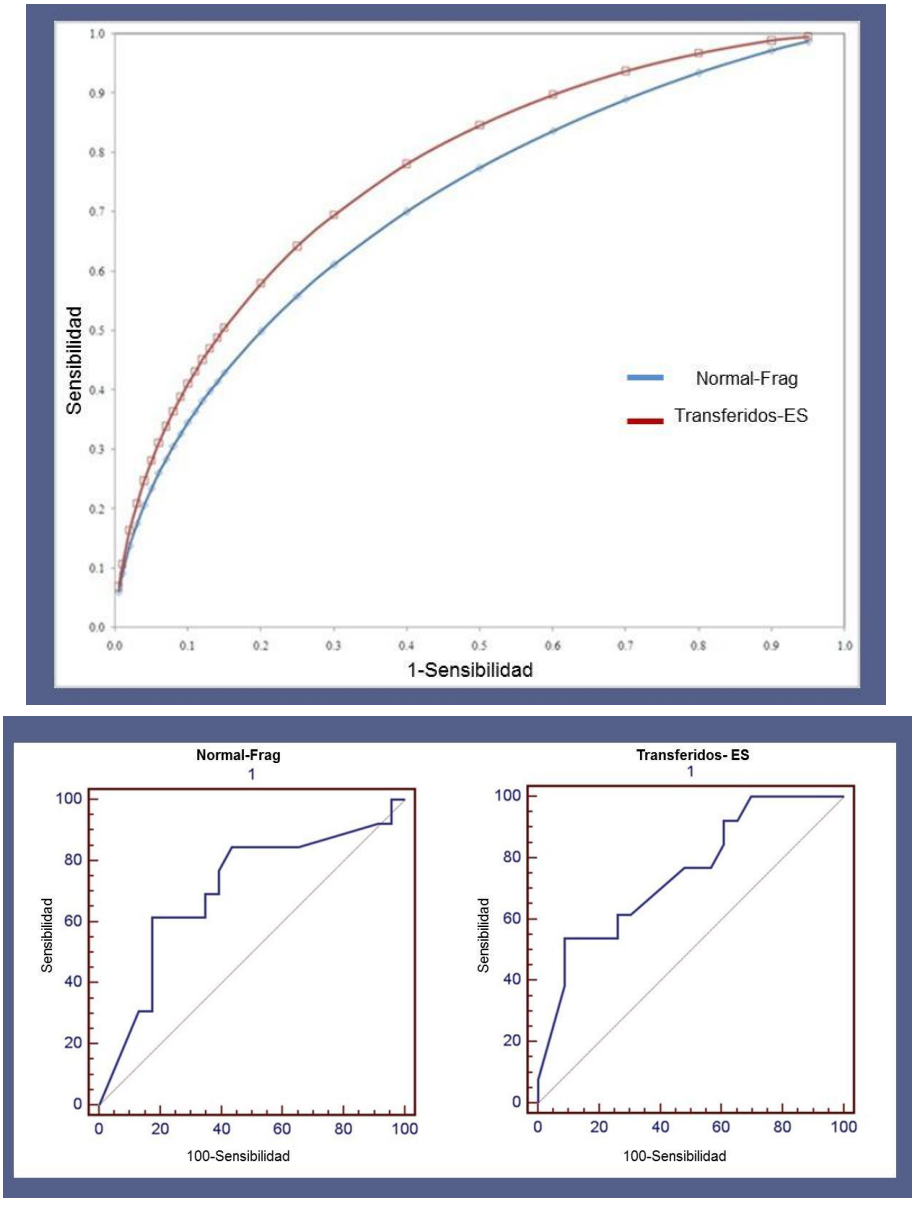


Figura 18. Curvas ROC para el porcentaje de espermatozoides normales con fragmentación de ADN (Normal-Frag) y la media de la calidad embrionaria de los embriones transferidos (CE-Transferidos) para predecir embarazo*. Cuantitativamente, el área bajo la curva es usada para determinar la precisión de predicción. El área bajo la curva ROC para Normal-Frag= 0,70 y para CE-transferidos= 0,72.*

Puesta a punto y validación de la técnica de TUNEL en espermatozoides de raton

Por motivos bioéticos, el estudio en modelos animales permite mayores posibilidades que el estudio en humanos. El modelo de ratón ha sido ampliamente utilizado dentro del Laboratorio de Reproducción de la Cátedra de Fisiología Humana de la Facultad de Ciencias Médicas de la Universidad Nacional de Córdoba. La puesta a punto de la técnica de TUNEL en el modelo de ratón brinda una nueva herramienta de análisis para futuros estudios dentro del grupo de trabajo.

Para permitir un control positivo adecuado se evaluó la fragmentación de ADN en los espermatozoides luego de tres tiempos de incubación y temperaturas de la DNasa. De acuerdo a los resultados se comprobó para lograr una correcta inducción de la fragmentación de ADN por la enzima, era necesario un tiempo de incubación de 30 minutos y una temperatura de 37 ° C. Los demás pasos en el procesado de las muestras de ratón fueron similares al procesado de espermatozoides humanos. La figura 19 muestra imágenes representativas de diferentes espermatozoides con el ADN fragmentado.

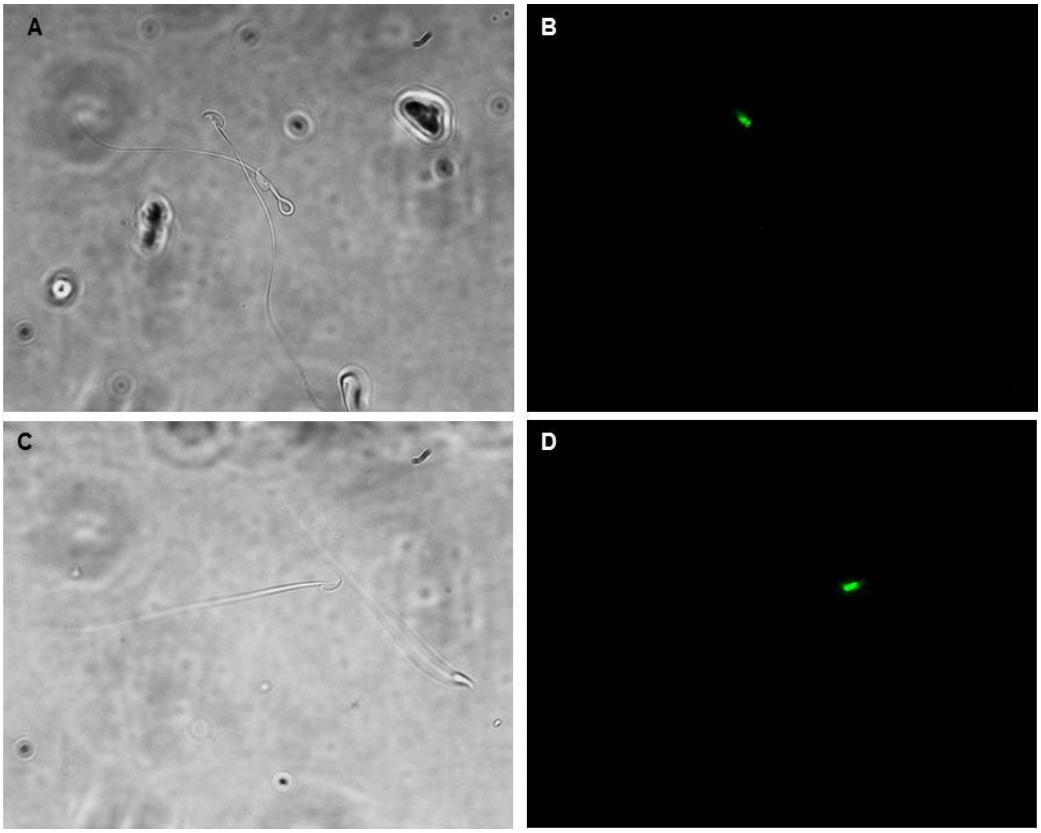


Figura 19. Fotomicrografía representativa de la evaluación de la fragmentación de ADN en espermatozoides de ratón obtenidos de epidídimos de machos adultos. *A y C: contraste de fase; B y D: fluorescencia. La fluorescencia indica fragmentación de ADN.*

Impacto en la integridad del ADN en espermatozoides de ratón alimentados con dieta enriquecida en ácidos grasos ω3

Mediante la utilización del ensayo de TUNEL se evaluó la integridad del ADN en espermatozoides epididimarios de ratón nutridos con alimento balanceado comercial suplementado con ω3 por tres meses. Los resultados fueron comparados con un grupo control de espermatozoides de ratones a los cuales se les dio alimento balanceado comercial sin el agregado de algún suplemente nutricional durante el mismo período de tiempo. No se observaron diferencias significativas en el porcentaje de espermatozoides con ADN íntegro entre los dos grupos estudiados (Figura 20).

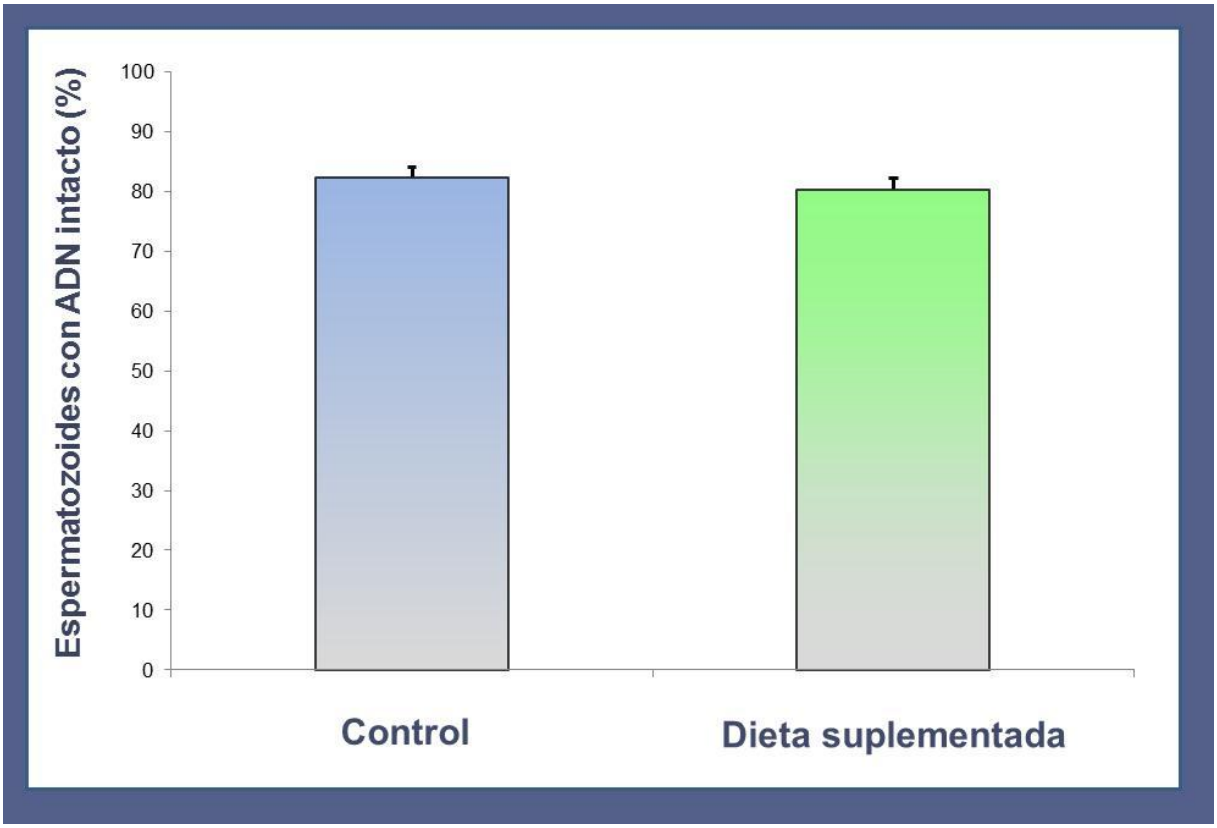


Figura 20. Incidencia de la suplementación dietaria con ω3 en espermatozoides epididimarios de ratón sobre la integridad del ADN. *Control: ratones (n=5) alimentados con alimento balanceado comercial; Dieta suplementada: ratones (n=5) alimentados con alimento balanceado comercial enriquecido con aceite de hígado de bacalao. Los resultados se expresan como media; las líneas verticales representan la desviación estandar. p< 0,05.*

DISCUSIÓN

DISCUSIÓN

Nuestros resultados, expuestos en este trabajo de tesis, ponen de manifiesto la necesidad de la utilización de espermatozoides con ADN íntegro en tratamientos de reproducción asistida. Hemos mostrado por primera vez la presencia de espermatozoides con morfología normal que presentaban fragmentación de ADN. Implementamos la evaluación simultánea de morfología y fragmentación de ADN espermática en la misma célula y, de esta manera, demostramos un claro efecto deletéreo en la utilización de espermatozoides con ADN fragmentado sobre la calidad embrionaria y la consecuente tasa de embarazo en tratamientos de reproducción asistida de alta complejidad (ICSI).

Los espermatozoides son células con características únicas. Son producidos en el cuerpo del macho pero su función la cumplen en un organismo diferente, la hembra. Son células altamente especializadas que tienen como principal objetivo transferir la información genética masculina dentro del ovocito de la mujer para producir un nuevo individuo genéticamente diferente a sus progenitores.

En el momento de la eyaculación, los espermatozoides son depositados, en la gran mayoría de los mamíferos, en la cavidad vaginal y para adquirir capacidad fertilizante deben experimentar diversos cambios estructurales y funcionales. La primera serie de eventos, que se inicia cuando la gameta masculina escapa del plasma seminal y que concluye momentos previos al encuentro con el ovocito, es conocido como *capacitación espermática*. Éste es un proceso en el cual ocurren un variado número de fenómenos necesarios para la normal ocurrencia del proceso de fertilización. Entre esos eventos, podemos mencionar cambios en la concentración de iones intracelulares, cambios en la membrana plasmática, incremento de AMP cíclico y fosforilación de proteínas, por nombrar solo los más estudiados. Cuando el espermatozoide alcanza el istmo del oviducto inicia un movimiento asimétrico, amplio y acelerado del flagelo (conocido como *hiperactivación*), lo que le permite alcanzar el ámpula, atravesar el *cúmulus ooforus* (células de la granulosa que rodean el ovocito) y encontrarse con la zona pelúcida donde es reconocido (reconocimiento entre gametos).

Al producirse la unión primaria entre la zona pelúcida y el espermatozoide, se desencadena la reacción acrosomal en varios puntos de la cabeza de la gameta

masculina, lo cual produce una fusión entre su membrana plasmática del espermatozoide y la membrana externa del acrosoma liberando enzimas hidrolíticas como la hialuronidasa, acrosina y tripsina que favorecen la penetración. Habiendo pasado a través de la zona pelúcida, el espermatozoide atraviesa el espacio perivitelino. Entonces, la cabeza del espermatozoide se une a la membrana plasmática del ovocito(ovolema) y todo el espermatozoide es incorporado al citoplasma del ovocito(ovoplasma). Tras la fusión con el espermatozoide, el ovocitoinicia a su vez una serie de eventos que conducen a la división y diferenciación de la célula y la formación de un nuevo individuo. Este proceso es conocido como *activación ovocitaria* e incluye los eventos de exocitosis de los gránulos corticales y la reanudación de la meiosis. En última instancia, ambos pronúcleos (masculino y femenino) se unen y fusionan para realizar *singamia*. El pronúcleo masculino está estrechamente asociado con el centrosoma, el cual organiza una matriz de microtúbulos (el áster) que se propaga a través del citoplasma del ovocito y de esa forma captura y atrae al pronúcleo femenino. Por otro lado, el áster creciente empuja contra la corteza del ovocito para mover ambos pronúcleos hacia el centro de la célula (Yanagimachi, 1994).

Para que un espermatozoide pueda cumplir la función para la que fue generado debe mostrar ciertas características. A saber: adecuado tamaño y forma, unión del cuello con la cabeza sin alteraciones detectables al microscopio, un acrosoma rodeando la porción superior de la cabeza y con las enzimas hidrolíticas necesarias para penetrar la zona pellucida y el ADN nuclear integro. Si por algún motivo, alguno de los elementos previamente mencionados no poseen sus características de normalidad, el espermatozoide no logrará (o lo hará anómalamente), fertilizar al ovocito. Por ejemplo, si el ADN se encuentra dañado, el espermatozoide podrá penetrar al ovocito, fertilizarlo y producir la singamia (Twigg et al, 1998). No obstante, el embrión generado por un espermatozoide con ADN dañado no podrá producir una normal embriogénesis (Menezo, 2006).

La introducción en los tratamientos de reproducción asistida de la técnica de inyección intracitoplasmática del espermatozoide (ICSI) ha brindado a muchos hombres infértiles con parámetros seminales severamente alterados, la posibilidad de la paternidad biológica. Sin embargo, ésta es una técnica más invasiva que la fertilización *in vitro* convencional ya que "evade" el proceso de selección natural del espermatozoide. En ICSI, solo un espermatozoide es seleccionado e inyectado dentro del citoplasma de un ovocito maduro y la selección del espermatozoide está basada

generalmente en las características de movilidad y morfología de la gameta, sin que el responsable de dicho proceso cuente con información precisa relativa al estado de los cromosomas o el ADN.

Por otro lado, algunos estudios sugieren que existe una mayor incidencia de anomalías cromosómicas en niños nacidos por ICSI en comparación con niños nacidos por concepción natural (Tarlatzis y Bili, 1998; Feng et al, 2008). Además, un aumento significativo en los problemas urogenitales de los bebés varones nacidos después de ICSI fue demostrado en un estudio realizado en Suecia (Wennerholm et al, 2000). Otros autores también han informado de una asociación entre defectos cardiovasculares, urogenitales, cromosómicos, y ósteo-mio-articulares en niños nacidos por técnicas de ICSI (Hansen et al, 2002).

El uso del ICSI como procedimiento de fertilización *in vitro* se ha convertido en el método más utilizado en tratamientos de reproducción asistida de alta complejidad. De acuerdo a la información del Centro para el Control y Prevención de Enfermedades (CDC, Center for Disease Control and Prevention, USA) en los Estados Unidos en el año 2010, el 66 % de los procedimientos de reproducción asistida de alta complejidad fueron por ICSI (CDC, 2012), mientras que en Europa la Sociedad Europea de Reproducción y Embriología Humana (ESHRE) ha informado que en el 69 % de los casos se ha realizado ICSI en el año 2008 (Ferraretti et al, 2012). Asimismo, en algunos países la proporción de procedimientos con ICSI supera el 90% de los tratamientos de reproducción asistida de alta complejidad (Ferraretti et al, 2012). Por otro lado, según datos de la Sociedad Argentina de Medicina Reproductiva (SAMER) en la Argentina durante el año 2008 el 86 % de los tratamientos fueron realizados por la técnica de ICSI (SAMER, 2012).

Las parejas con pobre calidad seminal y/o que hayan fallado tratamientos con fertilización *in vitro* convencional son, hoy en día, tratados con ICSI (Oehninger, 2001). Por otro lado, una gran variedad de estudios ha demostrado que hombres infértiles tienen altos niveles de fragmentación de ADN así como otros tipos de daños en el ADN en comparación con hombres fértiles (Zini et al, 2001; Benchaib et al, 2003; Sergerie et al, 2005). Hombres infértiles con diferentes grados de Oligo-asteno-teratozoospermia pueden presentar frecuentemente elevados valores de fragmentación de ADN; sin embargo, también se ha visto que pacientes infértiles con normozoospermia pueden tener altos niveles de daño en el ADN en comparación con hombres fértiles (Saleh et al, 2002). Por consiguiente, este grupo de hombres infértiles se enfrenta a la posibilidad de

tener mayor cantidad de espermatozoides con ADN dañado y a su vez que estos espermatozoides puedan ser seleccionados en el momento del ICSI.

Asimismo, estudios realizados en animales han demostrado que la utilización de espermatozoides con ADN dañado provocaría baja calidad embrionaria, disminución en las tasas de embarazo y hasta problemas en los recién nacidos. Se ha visto que espermatozoides de bovinos con ADN fragmentado pueden fertilizar un ovocito y completar los primeros ciclos de clivajes pero se bloquea el crecimiento en el estadío de blastocisto con inducción de apoptosis (Fatehi et al, 2006). Una disminución de la tasa de implantación y del desarrollo fetal ha sido demostrada cuando se utilizaron espermatozoides de ratón con ADN fragmentado (Pérez-Crespo et al, 2008). Fernández-González y col (2008) estudiaron el impacto en las crías de ratón nacidas luego de la realización de ICSI utilizando espermatozoides con ADN fragmentado; observaron un aumento en la ansiedad, falta de algunos patrones de habituación, déficit de memoria a corto plazo, órganomegalia y aparición de tumores en el grupo donde se utilizó espermatozoides con ADN dañado. Durante los últimos años, algunos autores han sugerido, en estudios realizados en humanos, que la utilización de espermatozoides con ADN dañado podría ser el causante de algunas de las anomalías congénitas mencionadas previamente y hasta podría ser el causante de mayores probabilidades de contraer cáncer en niños nacidos de padres con altos grados de fragmentación de ADN (Aitken et al, 2009).

Se han propuesto y utilizado un variado número de técnicas para el estudio de la integridad del ADN. Los métodos más utilizados son SCSA (del inglés *Sperm Chromatin Structure Assay*), el SCD (del inglés *Sperm Chromatin Dispersion*) o *Halosperm*®, el ensayo de Cometa y la técnica de TUNEL. Si bien metodológicamente estas técnicas son diferentes se ha mostrado una muy buena correlación entre los valores observados (Zini et al, 2001; Fernandez et al, 2003; Chohan et al, 2006). Sin embargo, en un estudio recientemente publicado se ha demostrado, utilizando gráficos de *Bland* y *Altman*, regresión de *Passing-Bablok* y correlación de concordancia, que estas evaluaciones no son comparables y que estas técnicas determinan diferentes aspectos del daño del ADN. Por un lado el SCSA evalúa el potencial daño del ADN en términos de susceptibilidad de desnaturalización del ADN mientras que el TUNEL evalúa el daño real del ADN (Henkel et al, 2010). Un importante aspecto de las técnicas para la determinación de fragmentación de ADN es que algunas (SCSA, SCD y ensayo de cometa a pH ácido o alcalino) requieren de un paso inicial de decondensación/desnaturalización con el fin

de detectar roturas en el ADN. Sin embargo, cuando el daño al ADN es observado bajo condiciones ácidas o alcalinas y no bajo condiciones neutras podríamos estar hablando de sitios de ADN lábiles a ácidos o álcalis. Por otro lado, el TUNEL y el ensayo de cometa a pH neutro no requieren de un paso inicial de decondesación y, por lo tanto, miden en daño al ADN directamente. El pH intracelular del ovocito es alrededor de 7,0 (Phillips y Baltz, 1996; Phillips et al, 2000), por lo que la presencia de sitios de ADN lábiles a ácidos o álcali podría no tener consecuencias significativamente negativas en la formación del pronúcleo masculino (Sakkas y Alvarez, 2010).

El ensayo de TUNEL es un método relativamente sencillo y se basa en la adición a cadenas de ADN rotas en una o dos de sus hebras, de un precursor del ADN (dUTP) marcado. Esta reacción es catalizada por la nucleotidil transferasa terminal (TdT) por medio de una reacción enzimática (Gorczyca et al, 1993). Ésta técnica puede ser medida tanto por citometría de flujo o por microscopia de fluorescencia. Por tener una alta sensibilidad y especificidad y una variabilidad intra e inter observador menor del 7 u 8% (Barroso et al, 2000) el ensayo de TUNEL es uno de los más utilizados en los estudios clínicos y ha sido propuesto por diferentes autores para ser incorporado dentro del análisis de semen convencional (Tarozzi et al, 2009; Sakkas y Alvarez, 2010).

Técnicamente, esta metodología se desarrolla en varias etapas: fijación, permeabilización e incubación con dUTP unido a algún fluorósforo (fluoresceína, rodamina, etc). El tiempo de procesado es entre 3 y 4 horas y luego las muestras deben ser analizadas por citometría de flujo o microscopía de fluorescencia, lo cual ha sido mencionado por algunos autores como una desventaja al momento de ser propuesto como un ensayo de rutina (Ozmen et al, 2007; Tarozzi et al, 2009) ya que es necesario aparatología compleja y personal capacitado. La evaluación por microscopia permite además estudiar la morfología espermática en la misma célula, lo cual aporta mayor información al ensayo.

Estudios de diferentes grupos de investigación realizados en humanos han demostrado que el aumento de espermatozoides con ADN fragmentado disminuye las chances de lograr un embarazo tanto de forma natural como por técnicas de reproducción asistida. Mediante diferentes métodos de análisis se ha mostrado que el daño en el ADN espermático está asociado con bajas probabilidades de lograr un embarazo de forma natural (Evenson et al, 1999; Spano et al, 2000; Loft et al, 2003; Sergerie et al, 2005; Giwercman et al, 2010; Sharma et al, 2010). También, altos valores

de fragmentación de ADN espermático han sido relacionados con bajas tasas de embarazo luego de inseminaciones intrauterinas (Duran et al, 2002; Muriel et al, 2006; Bungum et al, 2007). Por otro lado, se ha demostrado que el daño en el ADN espermático tiene relación con bajas tasas de fertilización, calidad embrionaria y/o tasas de embarazo luego de la realización de fertilización *in vitro* convencional (Filatov et al, 1999; Host et al, 2000; Benchaib et al, 2003; Henkel et al, 2003; Huang et al, 2005; Boe-Hansen et al, 2006; Borini et al, 2006; Bungum et al, 2007; Frydman et al, 2008; Lin et al, 2008; Tarozzi et al, 2009). Los datos obtenidos por Twigg (1998) y Henkel (Henkel et al, 2003; Henkel et al, 2004) sugieren que espermatozoides con ADN fragmentado son capaces de fertilizar a un ovocito, pero el desarrollo embrionario se detendría en el momento en que el genoma paterno se activa, lo que resultaría en una falla en el embarazo.

Por otro lado, en ICSI los resultados de diferentes grupos de trabajo son contradictorios. Mientras un número de autores han mostrado una influencia negativa en los resultados de fertilización y embarazo en pacientes con altos porcentajes de espermatozoides con ADN fragmentado (Lopes et al, 1998; Henkel et al, 2003; Tesarik et al, 2004; Benchaib et al, 2007; Tavalaee et al, 2009), otros no han detectado ningún efecto (Lin et al, 2008; Simon et al, 2010; Esbert et al, 2011; Thomson et al, 2011).

Tavalaee y col (Tavalaee et al, 2009) estudiaron mediante la técnica de SCD, el impacto de la fragmentación de ADN en el semen de 66 pacientes que realizaron el procedimiento de ICSI. Los autores encontraron una correlación negativa entre el porcentaje de fragmentación de ADN y la tasa de fertilización pero no pudieron encontrar correlación con la tasa de clivaje, la calidad embrionaria ni con la tasa de embarazos. Por su parte, estudios realizados por Benchaib y col (Benchaib et al, 2007) utilizaron en ensayo de TUNEL para evaluar 234 muestras de semen. El análisis se realizó entre 2 a 5 meses previos al procedimiento de ICSI. Ellos observaron una disminución de la tasa de fertilización a medida que aumentaba la cantidad de espermatozoides fragmentados. Por otro lado, cuando la fragmentación de ADN era mayor a 15 % aumentaba el riesgo de pérdidas del embarazo. Henkel y col (Henkel et al, 2003) investigaron el impacto de la fragmentación de ADN (mediante TUNEL) sobre los resultados de ICSI en 54 pacientes. Si bien encontraron diferencias en las tasas de embarazos entre las parejas que presentaban menor proporción de espermatozoides con ADN fragmentado y las que tenían una alta proporción, estas diferencias no fueron significativas.

Lopes y col (Lopes et al, 1998) estudiaron 150 muestras utilizadas en tratamientos de ICSI utilizando la técnica de TUNEL. Mostraron una correlación negativa entre el porcentaje de espermatozoides con ADN fragmentado y la tasa de fertilización pero no hubo correlación con la tasa de clivaje embrionario. También, el grupo de Tesarik y col (Tesarik et al, 2004) evaluó 18 pacientes que habían fallado en intentos de FIV y que realizaron ICSI. Ellos concluyeron que el efecto paterno temprano no está afectado por el aumento de la fragmentación de ADN (evaluada por TUNEL) y que el daño en el ADN está relacionado con un efecto paterno tardío no asociado a la calidad de los embriones ni a la tasa de clivaje pero si a la tasa de implantación.

Contrariamente, Esbert y col (Esbert et al, 2011) evaluaron fragmentación de ADN por TUNEL en 116 muestras de pacientes que realizaban ICSI. Los resultados no mostraron relación entre la tasa de fertilización y la fragmentación de ADN, o entre en la calidad embrionaria y la fragmentación de ADN. Asimismo no hubo diferencias en la fragmentación de ADN entre los grupos de pacientes que lograron y los que no lograron el embarazo. Thomson y col (Thomson et al, 2011) evaluaron la fragmentación de ADN (TUNEL) en 48 muestras de espermatozoides que fueron utilizadas para la realización del ICSI; no encontraron diferencias significativas entre el grupo que embarazó y el que no embarazó. Sin embargo, dentro de este estudio los autores incluyeron no solo infertilidad por factor masculino sino otras causas (endometriosis, obstrucciones tubarias, etc). Estudios realizados por Simon y col (Simon et al, 2010) no mostraron relación entre fragmentación de ADN y los resultados de ICSI en 130 pacientes. Ellos utilizaron el ensayo de cometa en muestras destinadas a ICSI.

Lin y col (Lin et al, 2008) estudiaron 86 muestras de pacientes que serían sometidos a ICSI. La fragmentación de ADN se analizó por SCSA en una muestra de semen entero. No encontraron diferencias significativas en la tasa de fertilización, calidad embrionaria y tasa de embarazo entre grupos de pacientes con alto, moderado y bajo porcentaje de espermatozoides con fragmentación de ADN.

Estas discrepancias podrían deberse a que los estudios generados por los diferentes grupos son muy heterogéneos en términos de diseño experimental, los criterios de exclusión/inclusión o la metodología para evaluar la fragmentación del ADN espermático. Por otro lado, es importante tener presente que en el procedimiento de ICSI los espermatozoides son seleccionados de acuerdo a sus características de movilidad y de aspecto (morfología). Únicamente las células móviles y con morfología

normal serán seleccionadas por el embriólogo/a en el momento de la inyección del espermatozoide dentro del citoplasma del ovocito (Palermo et al, 1992). Además, los espermatozoides con ADN dañado no pueden ser reconocidos durante el procedimiento de selección, por lo que la inyección inadvertida de espermatozoides con este defecto podría ser también la causa de estas discordancias.

Estos antecedentes (principalmente los estudios en modelos animales) ponen de manifiesto la importancia de la evaluación y utilización de espermatozoides con ADN integro en procedimientos de reproducción asistida. El punto de partida de este trabajo de tesis fue la evaluación de la integridad del ADN espermático en las muestras utilizadas para ICSI.

Utilizando la técnica de TUNEL se evaluó una fracción de espermatozoides de la misma muestra que se utilizó para ICSI. Los resultados mostraron que no hay diferencias estadísticamente significativas en el porcentaje de espermatozoides con fragmentación de ADN entre el grupo de pacientes que embarazaron y los que no lograron el embarazo. Tampoco hubo correlación entre la cantidad de espermatozoides TUNEL positivo y la calidad embrionaria o la tasa de fertilización. Nuestros resultados fueron similares a los publicados por otros autores (Lin et al, 2008; Simon et al, 2010; Esbert et al, 2011; Thomson et al, 2011) y sugieren que la evaluación de la integridad del ADN en el total de los espermatozoides no tendría impacto sobre los resultados de ICSI. Es por esta razón y dado que en el semen coexisten una gran variedad de subpoblaciones de espermatozoides con diferentes morfologías (Menkveld et al, 1990; WHO, 1999) y , por lo antes mencionado, para la realización de la técnica de ICSI solo los espermatozoides con morfología normal son seleccionados e inyectados. Con lo cual, la evaluación de la fragmentación de ADN en el total de subpoblaciones espermáticas no necesariamente podría impactar en el resultado de la técnica de ICSI.

A partir de estos resultados, se estudió entonces la incidencia de fragmentación de ADN en espermatozoides móviles con diferentes patrones morfológicos. De 24 muestras de semen de pacientes infértiles, en los cuales se evaluó la morfología de los espermatozoides que presentaban fragmentación de ADN. De las 7.595 células estudiadas, el 98,1% presentaban alguna anomalía morfológica, mientras que solo el 1,9% eran espermatozoides morfológicamente normales. Se observó que la mayor proporción de espermatozoides con ADN fragmentado (45%) tenían un patrón de morfología que correspondía a espermatozoides amorfos. Éstos resultados reafirman el

hecho que la evaluación de la fragmentación de ADN en el total de espermatozoides claramente subestima el impacto que podría tener la integridad del ADN espermático en la técnica de ICSI.

Ha sido previamente demostrado que hombres infértiles con diferentes anomalías en el semen tienen mayor grado de fragmentación de ADN espermático que hombres fértiles. Se ha visto que espermatozoides con baja movilidad tienen mayor incidencia de daño en el ADN. Por medio de separación espermática con gradientes, se ha demostrado que en espermatozoides de pacientes infértiles con baja movilidad mostraban mayor proporción de fragmentación de ADN que los espermatozoides con alta movilidad en la misma muestra (Barroso et al, 2000; Weng et al, 2002). Gandini y colaboradores (Gandini et al, 2000) evaluaron la relación entre fragmentación de ADN y parámetros seminales y observaron una correlación negativa con la concentración y la movilidad espermática. Esta correlación fue aún mayor cuando se comparó con la morfología. Por otro lado, se ha visto que hombres con oligo-asteno-teratozoospermia pueden presentar mayores grados de fragmentación de ADN en comparación con hombres normozoospérmicos. El grupo de Huang y col (Huang et al, 2005) utilizaron el ensayo de TUNEL para evaluar la correlación entre fragmentación de ADN y los parámetros seminales. Los autores encontraron un aumento significativo de la fragmentación de ADN en pacientes con parámetros seminales alterados comparados con pacientes con parámetros seminales normales. Sin embargo, algunos estudios han mostrado que hombres infértiles con parámetros seminales normales (normozoospérmicos) pueden tener mayor número de espermatozoides con ADN fragmentado en comparación con hombres fértiles y normozoospérmicos (Saleh et al, 2002; Saleh et al, 2003).

A partir de estas evidencias, se evaluó la fragmentación de ADN en el total de los espermatozoides y en la subpoblación de espermatozoides morfológicamente normales y móviles de tres grupos: hombres fértiles, hombres que consultaban por primera vez por infertilidad y pacientes cuyo diagnóstico de infertilidad era de causa masculina (teratozoopermia); éstos últimos serían tratados con ICSI. Tal como lo han demostrado otros autores, cuando se evaluó el total de espermatozoides y sin importar la morfología (anormal o normal), se encontró un claro aumento de espermatozoides con fragmentación de ADN en el grupo de los pacientes infértiles comparado con los hombres fértiles. Por otro lado, cuando se evaluó la fragmentación de ADN en los espermatozoides morfológicamente normales, ninguno de los hombres fértiles mostró

espermatozoides dañados, mientras que en solo un paciente subfertil se encontró espermatozoides normales con ADN fragmentado. Sin embargo, en todas las muestras de pacientes infértiles se encontraron espermatozoides morfológicamente normales con fragmentación de ADN.

Según nuestro conocimiento y experiencia, éste es el primer estudio en examinar simultáneamente los espermatozoides de morfología normal y la fragmentación de ADN. Se ha demostrado por primera vez la presencia de la fragmentación ADN en espermatozoides con morfología aparentemente normal. Los resultados demostraron la ausencia de fragmentación de ADN en espermatozoides normales de hombres fértiles y que en hombres infértiles con severa o moderada teratozoospermia los niveles de daño en el ADN eran significativamente más elevados.

Los espermatozoides móviles y morfológicamente normales son las células que tienen alta probabilidad de ser seleccionados por el embriólogo/a en el momento de la inyección de ovocitos para ICSI y en consecuencia investigamos el impacto de la evaluación de espermatozoides normales con ADN fragmentado sobre los resultados de ICSI, medidos en términos de la calidad embrionaria y el potencial de embarazo. Se seleccionaron parejas que presentaban infertilidad exclusivamente por factor masculino y no tenían evidencias de factor femenino que pudiera disminuir las chances de embarazo.

Nuestro estudio muestra una correlación negativa y estadísticamente significativa entre el porcentaje de espermatozoides morfológicamente normales con ADN fragmentado y la calidad embrionaria medida como la calidad media del total de embriones después de ICSI. Es importante destacar que los resultados también confirman que esta asociación se mantuvo al analizar sólo los embriones transferidos. El porcentaje de espermatozoides normales con fragmentación de ADN tuvo una correlación negativa y estadísticamente significativa con la calidad de los embriones transferidos. Estos resultados apoyan el hecho de que la presencia de espermatozoides normales con fragmentación de ADN tiene un importante impacto negativo en la calidad de los embriones después del ICSI. Contrariamente a otros estudios (Tesarik et al, 2004; Borini et al, 2006), no encontramos una correlación estadísticamente significativa entre la fragmentación de ADN espermática total y la calidad embrionaria, lo que sugiere que el análisis de la subpoblación de espermatozoides morfológicamente normales mejora la capacidad de predecir el impacto de la fragmentación del ADN

espermático en la calidad del embrión. Esto fue apoyado por el análisis de regresión múltiple, que reveló que el porcentaje de espermatozoides normales con fragmentación de ADN, la edad de la mujer y el número de ovocitos fertilizados fueron los principales factores determinantes de la calidad de los embriones.

Otro hallazgo importante fue que cuando se realizó el análisis de la curva ROC, el porcentaje de espermatozoides morfológicamente normales con fragmentación de ADN y el promedio de la calidad de los embriones transferidos fueron predictores estadísticamente significativos de embarazo. Según lo estimado por el análisis de la curva ROC y el uso de valores de corte óptimos, ambos parámetros mostraron una alta especificidad y fueron predictores confiables de la concepción. En general, se estima que una prueba con una razón de verosimilitud (LR) mayor de 10 predice de forma concluyente el resultado (en este caso el embarazo), mientras que con una LR entre 5 a 10 es un moderado y un LR entre de 2 a 5 es un predictor débil de resultado (McGee, 2002). De acuerdo con estos criterios, se concluye que la calidad de los embriones transferidos es un moderado predictor (LR: 6,2) y el porcentaje de espermatozoides morfológicamente normales con fragmentación de ADN es un débil predictor (CP: 3,5) de embarazo en ciclos de ICSI.

El porcentaje de espermatozoides morfológicamente normales con fragmentación de ADN predice indirectamente el embarazo a través la calidad de los embriones transferidos, lo que está indicado por su fuerte correlación y por el hecho de que en el análisis de regresión logística sólo la calidad de los embriones transferidos se incluyó en el modelo estadístico predictivo de la concepción. Además, cuando la medida de la calidad de los embriones transferidos fue excluida de la lista de los parámetros evaluados, el modelo estimado para predecir el embarazo sólo incluyó el porcentaje de espermatozoides morfológicamente normales con fragmentación de ADN. Este hallazgo apoya la conclusión de que el porcentaje de espermatozoides normales con fragmentación de ADN predice indirectamente el embarazo a través de la calidad de los embriones transferidos.

Para lograr un embarazo es necesario que tanto el espermatozoide y el ovocito, como el endometrio estén en buenas condiciones. En nuestro estudio hemos descartado los pacientes que presentaban algunos de los factores que podrían afectar la calidad embrionaria y el embarazo como por ejemplo la edad de la mujer o la cantidad de ovocitos recuperados luego de la aspiración ovocitaria. Sin embargo, al presente es

imposible estudiar de forma certera la calidad endometrial y por lo tanto la probabilidad de saber si el endometrio será receptivo (Revel, 2012). Estas razones podrían ser las causas de que la evaluación de la fragmentación de ADN en espermatozoides morfológicamente normales sea un débil predictor de embarazo y un moderado predictor de calidad embrionaria.

Todas las células, excepto los virus y los espermatozoides poseen una variedad de mecanismos enzimáticos que reparan el ADN dañado (Ashwood-Smith y Edwards, 1996). Los sistemas de reparación del ADN están implicados en la recombinación de genes a través de la formación de quiasmas y entrecruzamientos cromosómicos en la meiosis. Existe evidencia experimental en sistemas *in vivo* e *in vitro* que indican claramente que el ovocito de vertebrados es capaz de reparar el ADN endógena y exógenamente dañado (Ashwood-Smith y Edwards, 1996). Los ovocitos de ratón tienen la capacidad de reparar el ADN dañado de los espermatozoides (Derijck et al, 2008), aunque esta capacidad es limitada y puede variar entre ovocito y ovocito del mismo o diferente ratón. Esto también podría depender de la edad de la mujer. Por otro lado, la habilidad del ovocito de reparar el ADN dañado del espermatozoide que lo fertiliza, también dependerá del tipo de daño en el ADN. En general, si el daño es en una sola de las cadenas de ADN, éste será mucho más fácil de reparar que si el daño es en las dos cadenas, aunque se ha visto que las polimerasas pueden también reparar ADN dañado en las dos hebras (Garcia-Diaz et al, 2000). Por lo tanto, si un espermatozoide con ADN dañado logra fertilizar un ovocito, éste podría repararlo y por consiguiente no tener consecuencias para el embrión y el desarrollo fetal. Sin embargo, hasta el momento, no podemos determinar si el ovocito utilizado tendrá la capacidad de reparar el ADN. Asimismo, recientemente se ha demostrado que la mayoría de las roturas en el ADN del espermatozoide persisten después de la fertilización y que la activación de la síntesis de ADN en el cigoto no repara el genoma paterno dañado (Yamauchi et al, 2012). Además, nuevas evidencias han demostrado que los ovocitos fertilizados con espermatozoides con ADN dañado, producen cigotos que demoran la activación del genoma paterno y como última instancia conducen a la detención del desarrollo embrionario (Gawecka et al, 2013).

Estudios previos han revelado que muestras con más del 15% o 20% de espermatozoides con ADN fragmentado (evaluado por TUNEL) presentan menos chances de lograr un embarazo que muestras con valores inferiores (Benchaib et al, 2003; Borini et al, 2006).

Sin embargo, nuestros resultados mostraron que la evaluación del total de espermatozoides con ADN fragmentado no estaría asociada con los resultados del ICSI. En cambio, la proporción de espermatozoides morfológicamente normales con ADN fragmentado es un buen predictor de la calidad embrionaria y un predictor débil de embarazo. Estos hallazgos sugieren que muestras seleccionadas por *swim-up* que contengan más de 17,6% de espermatozoides morfológicamente normales con ADN fragmentado estarían asociadas con una mayor posibilidad de generar embriones de mala calidad y con menos probabilidades de resultar en un embarazo.

Algunos estudios han investigado la relación entre pérdida del embarazo y el daño en el ADN espermático (Bungum et al, 2004; Virro et al, 2004; Check et al, 2005; Benchaib et al, 2007; Bungum et al, 2007; Ozmen et al, 2007; Frydman et al, 2008; Lin et al, 2008; Esbert et al, 2011) y han concluido que la fragmentación del ADN está asociado con un aumento significativo del riesgo de pérdida del embarazo, tanto por concepción espontánea así como luego de la realización de una FIV convencional o un ICSI (Zini et al, 2008; Robinson et al, 2012). En nuestro estudio no hemos tenido pérdidas de embarazos, por lo cual no es posible arriesgar alguna opinión al respecto; es posible que considerando un mayor número de muestras evaluadas se pueda detectar si existe algún tipo de asociación.

Por último, con el fin de incorporar el estudio de la integridad del ADN espermático en espermatozoides de ratón, se puso a punto la técnica de TUNEL para espermatozoides epididimarios murinos. Se desarrolló un estudio base donde se comparó la fragmentación de ADN en espermatozoides de ratones alimentados con una dieta enriquecida con omega 3 (ω3). Se ha propuesto que las dietas ricas en ω3 tendrían un efecto beneficioso sobre la espermatogénesis y la calidad de los espermatozoides, tanto en humanos como en animales (Roqueta-Rivera et al, 2011; Safarinejad, 2011; Yan et al, 2013). Sin embargo los resultados aquí presentados no mostraron diferencias significativas entre los ratones con dieta suplementada y los ratones controles. Estas discrepancias podrían deberse a que el tiempo de administración (3 meses) no fue suficientemente prolongado o, bien, el número de especímenes estudiados fue escaso para observar cambios en la integridad del ADN. De todas formas, estos estudios iniciales brindan la base para la aplicación de la evaluación de la fragmentación de ADN espermática en uno de los modelos animales más utilizados en el Laboratorio de Reproducción de la Cátedra de Fisiología Humana de la Facultad de Ciencias Médicas de la Universidad Nacional de Córdoba.

Conclusiones

En base a los resultados previamente discutidos, es posible concluir que:

- Este trabajo de tesis aporta nuevas evidencias relativas a la técnica de TUNEL para el estudio de la fragmentación del ADN en espermatozoides humanos y su eficacia en la predicción de éxito de los resultados de la técnica de ICSI.
- Por primera vez se ha mostrado la presencia de fragmentación de ADN en espermatozoides morfológicamente normales de hombres infértiles con severa o moderada teratozoospermia mientras no hemos encontrado células normales con ADN dañado en hombres fértiles.
- Existe una asociación estadísticamente significativa entre espermatozoides con ADN fragmentado y los resultados de ICSI.
- La evaluación de la fragmentación de ADN espermático sin prestar atención a la morfología de la gameta no muestra correlación con los resultados de ICSI.
- La evaluación de la fragmentación de ADN en espermatozoides morfológicamente normales correlaciona negativamente con la calidad embrionaria.
- La evaluación de la fragmentación de ADN en el total de espermatozoides claramente subestima el impacto que podría tener la integridad del ADN espermático en la técnica de ICSI.
- Identificamos un límite de corte para predecir éxitos en los tratamientos de reproducción asistida de alta complejidad.
- Hemos logrado desarrollar una nueva forma de estudiar la integridad del ADN evaluando en forma simultánea la subpoblación de espermatozoides morfológicamente normales.
- Si bien no es posible determinar la integridad del ADN en los espermatozoides que serán inyectados durante el procedimiento de ICSI,

nuestros resultados sugieren que la evaluación del daño en el ADN en espermatozoides morfológicamente normales es la aproximación más confiable para reflejar el impacto de estos espermatozoides sobre la calidad embrionaria y las probabilidades de lograr un embarazo.

- Por último, hemos logrado un éxito aceptable en la aplicación de la técnica de TUNEL en espermatozoides de ratón con el equipo del Laboratorio de Reproducción de la Cátedra de Fisiología Humana de la Facultad de Ciencias Médicas de la Universidad Nacional de Córdoba.

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ANEXO I

Instituto y Cátedra de Fisiología Humana
Facultad de Ciencias Médicas Universidad Nacional de Córdoba

CERTIFICO que los resultados en animales de experimentación (ratones alimentados con dietas con ofertas diferentes de ácidos grasos) incluidos en la Tesis Doctoral "**Fragmentación del ADN espermático en pacientes infértiles y su correlación con técnicas de reproducción asistida de alta complejidad (ICSI)**" fueron obtenidos mediante experimentos realizados en el Laboratorio de Reproducción de esta Unidad Académica.----

Con el propósito de incorporar esta técnica a la línea experimental que explora la actividad funcional espermática de mamíferos, el Bioq. Conrado Avendaño obtuvo resultados preliminares necesarios para la validación del procedimiento en nuestro modelo en roedores.----------

Por el presente autorizo a su vez que los referidos valores sean incluidos y discutidos en su Tesis para optar al título de Doctor en Ciencias de la Salud.----------

A los fines que hubiere lugar, se extiende el presente a dieciséis días del mes de septiembre de dos mil trece.----------

Dra. Graciela Stutz
Profesora Encargada
(En cargo de Prof. Titular DE)

CATEDRA DE FISIOLOGIA HUMANA
UNIVERSIDAD NACIONAL DE CORDOBA
FACULTAD DE CIENCIAS MEDICAS

PROF. DRA. GRACIELA STUTZ
CATEDRA E INST. DE FISIOLOGÍA
FACULTAD DE CS. MEDICAS U.N.C.

Santa Rosa 1085 - CP: X5000ESU - Córdoba, Argentina - Telefax: (54) 351 - 4332019
e-mail: fisiolog@mater.fcm.unc.edu.ar web:www.fcm.unc.edu.ar/fisio

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Telephone (757) 446-7100 Fax (757) 446-7455 / (757) 446-8998
729 Thimble Shoals Boulevard, Suite 5E, Newport News, Virginia 23606
Telephone (757) 599-9893
www.JonesInstitute.org

19 de Marzo, 2009

A quien corresponda:

Por la presente dejo constancia que el Sr. Bioquímico Conrado Avendaño, bajo mi supervisión, se ha desempeñado como investigador científico en el *Jones Institute for Reproductive Medicine* dependiente del departamento de ginecología y obstetricia del *Eastern Virginia Medical School* (EVMS, Norfolk, Virginia, USA), realizando estudios sobre "Fragmentación de ADN en espermatozoides de pacientes candidatos a ICSI".

Dicho estudio fue subsidiado por la *Jones Institute Foundation* y aprobado por el Comité de Bioética de EVMS (*The Institutional Review Board of Eastern Virginia Medical School*).

Asimismo, por la presente AUTORIZO al señor Conrado Avendaño a incluir los resultados de ésta línea de investigación en su Tesis Doctoral para optar al título de Doctor en Ciencias de la Salud en la Facultad de Ciencias Médicas de la Universidad Nacional de Córdoba, Argentina y ACEPTO desempeñarme en dicho trabajo como Co-Director.

Sin otro particular, atentamente,

Sergio Oehninger, MD, PhD
Professor and Vice-Chair, Department of OB/GYN;
Professor of Urology;
Director, Division of Reproductive Endocrinology and Infertility
The Jones Institute for Reproductive Medicine
Eastern Virginia Medical School

ANEXO II

Los resultados surgidos de esta tesis doctoral fueron presentados y/o publicados en los siguientes lugares.

Publicaciones

- **Avendaño C** and Oehninger S. "DNA fragmentation in morphologically normal spermatozoa: how much should we be concerned in the ICSI era?". Review. Journal of Andrology, 2011. Jul-Aug;32(4):356-63.

- **Avendaño C**, Franchi A, Duran H, Oehninger S. "DNA fragmentation of normal spermatozoa negatively impacts embryo quality and ICSI outcome". Fertility & Sterility, 2010, Jul;94(2):549-57.

- Barroso G, Valdespin C, Vega E, Kershenovich R, Avila R, **Avendaño C**, Oehninger S. "Developmental sperm contributions: fertilization and beyond". Review. Fertility & Sterility, 2009, Sep;92(3):835-48.

- **Avendaño C**, Franchi A, Taylor S, Morshedi M, Bocca S, Oehninger S. "Fragmentation of DNA in morphologically normal human spermatozoa". Fertility & Sterility. 2009 Apr;91(4):1077-84.

MALE FACTOR

Fragmentation of DNA in morphologically normal human spermatozoa

Conrado Avendaño, M.Sc., Anahí Franchi, Ph.D., Steven Taylor, Ph.D., Mahmood Morshedi, Ph.D., Silvina Bocca, M.D., Ph.D., and Sergio Oehninger, M.D., Ph.D.

Department of Obstetrics and Gynecology, Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, Norfolk, Virginia

Objective: To evaluate DNA fragmentation in spermatozoa with normal morphological appearance.
Design: Prospective study.
Setting: Academic tertiary center.
Patient(s): Fertile, subfertile, and infertile men were studied.
Intervention(s): Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–fluorescein nick-end labeling assay and morphology assessment by phase contrast in the swim-up fractions.
Main Outcome Measure(s): Simultaneous assessment of the percentage of normally shaped sperm and DNA fragmentation.
Result(s): No DNA fragmentation was found in spermatozoa with normal morphology in any of the samples from the fertile group. In only one sample from the subfertile group did we observed normally shaped sperm cells exhibiting DNA fragmentation. However, in all the samples from the infertile group, we observed normal spermatozoa with DNA fragmentation. Spermatozoa from this late group exhibited a high proportion of DNA damage.
Conclusion(s): In infertile men with moderate and severe teratozoospermia, the spermatozoa with apparently normal morphology present in the motile fractions after swim-up may have DNA fragmentation. (Fertil Steril® 2009;91:1077–84. ©2009 by American Society for Reproductive Medicine.)

Key Words: DNA fragmentation, normal morphology, sperm selection, teratozoospermia

Sperm morphology has been recognized as an excellent predictor of the outcome of in vivo natural conception (1), intrauterine insemination (2) and conventional IVF therapies (3–5). In addition, the morphological normalcy of the sperm nucleus has been established as an important factor for achieving pregnancy after intracytoplasmic sperm injection (ICSI), as demonstrated recently by the use of high-magnification methods of sperm selection for microinjection (6, 7).

Ejaculated spermatozoa from infertile men reveal a variety of alterations of chromatin organization and structure, single-strand or double-strand DNA breaks, aneuploidy, and/or chromosome Y microdeletions (8, 9). Among such abnormalities, DNA damage, particularly in the form of DNA fragmentation, appears to be one of the main causes of decreased reproductive capacity of men, both in natural fertility as well as in assisted conception. Patients with oligoasthenoteratozoospermia, who more frequently require ICSI to overcome their infertile condition, have an increased sperm aneuploidy rate, despite a normal blood karyotype and increased levels of DNA fragmentation. Additional studies have shown that couples in whom pregnancy resulted in miscarriage demonstrated a trend toward poorer sperm DNA integrity, compared with fertile couples (10–14).

The prevalence of chromosomal abnormalities (de novo abnormalities) was found to be significantly higher among children conceived through ICSI than among naturally conceived children (15). Epigenetic abnormalities, such as errors in DNA methylation, have been linked to certain rare genetic diseases (Beckwith-Wiedemann and Angelman's syndromes) and, although still rare, they are found in slightly higher numbers among children conceived through IVF-ICSI than among naturally conceived children (16).

Because the presence of a high percentage of spermatozoa with DNA damage may have a negative effect on the outcome of assisted reproductive technologies (17), the exclusion of spermatozoa with nuclear defects thus can be expected to decrease the probability of accidental injection of a DNA-damaged spermatozoon into the oocyte. Intracytoplasmic

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Reprint requests: Sergio Oehninger, M.D., Ph.D., Department of Obstetrics and Gynecology, Jones Institute, Eastern Virginia Medical School, 601 Colley Avenue, Norfolk, Virginia 23507 (FAX: 757-446-8998; E-mail: oehninsc@evms.edu).

sperm injection bypasses natural sperm selection processes, because the embryologist subjectively chooses the spermatozoon to be injected into the oocyte on the basis of its motility and morphologic appearance. However, these selection criteria will not exclude, for example, the presence of a chromosomal abnormality, acquired during spermatogenesis as a result of an altered intratesticular environment, that may disrupt the fine-tuned mechanisms of chromosome segregation during spermatogenesis. Of particular concern is the fact that spermatozoa with normal morphology also may be aneuploid, canceling out the benefit of the careful selection of a normally shaped spermatozoon for ICSI, which is a process intended to reduce the risk of transmitting aneuploidy to the ICSI offspring (18, 19).

It is believed that if the genetic damage in the male germ is severe, embryonic development may arrest at the time that the paternal genome is switched on, resulting in a failed pregnancy (12, 17, 20). However, genetic and biological protection mechanisms do not necessarily preclude further embryonic development, because fertilization with damaged spermatozoa can result in a live-born infant (20, 21). In addition, reports regarding increased chromosomal abnormalities, minor or major birth defects, or childhood cancer suggest increased risks for babies born after ICSI (15, 17).

The findings mentioned in the previous paragraph suggest that paternal genomic alterations may compromise not only fertilization and early embryo quality but also embryo development and progression of pregnancy, resulting in spontaneous miscarriage. To date, a number of studies have highlighted the potential influence of a so-called paternal factor, but the relationship between sperm DNA integrity and early postimplantation embryo development in couples who are undergoing assisted reproductive techniques remains to be fully understood (12, 17, 20, 22).

The aims of this study were as follows: [1] to evaluate DNA integrity, as assessed by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–fluorescein nick-end labeling (TUNEL) assay, in spermatozoa from fertile, subfertile, and infertile men and [2] to determine the incidence of DNA fragmentation in spermatozoa with normal morphology that were obtained from the separated fractions of highly motile sperm, because these are the cells that have a high probability of being chosen by the embryologist at the time of oocyte injection for ICSI.

MATERIALS AND METHODS

Subjects

This was a prospectively designed clinical study. The Institutional Review Board of Eastern Virginia Medical School approved the study, and all participants gave written informed consent. Ejaculates from 19 men were studied. The participating individuals were classified into three groups: [1] a fertile group (FER, n = 4), which included healthy male volunteers without any history of infertility problems and whose partners had conceived and delivered a child within the last 2 years; [2] a subfertile group (SF, n = 5), including men being evaluated for infertility (defined as the inability to achieve a pregnancy in a stable relationship for at least a 1-year period); and [3] an infertile group (INF, n = 10), which included patients participating in our ICSI program who had a diagnosis of male infertility associated with teratozoospermia and who had failed controlled ovarian hyperstimulation combined with intrauterine insemination therapy in the absence of female factors (23).

Sperm Preparation

Semen samples were collected by masturbation into sterile cups after 2–4 days of sexual abstinence. The samples were allowed to liquefy for 30 minutes at room temperature, followed by assessment of semen characteristics and sperm parameters. Sperm concentration and progressive motility were assessed by using an HTR-IVOS semen analyzer (Hamilton Thorne Research, Beverly, MA) and were manually monitored, with fixed parameter settings (24). Motion parameters were examined after mixing the sperm suspension and loading a 5-μL aliquot into a Makler chamber (MidAtlantic Diagnostics Inc., Mt. Laurel, NJ). Sperm morphology was examined by using $\times$1,000 oil immersion microscopy, using strict criteria (3, 4, 25), as described elsewhere in reports from our laboratories (4, 5), after staining with STAT III Andrology Stain (MidAtlantic Diagnostics Inc.). Teratozoospermia was classified as severe ($\leq$4% normal forms or "poor prognosis pattern") or as moderate (5%–10% normal forms), in the same way as published elsewhere in reports from our laboratory (4, 5).

Motile spermatozoa were selected by swim-up that was performed in human tubal fluid (Irvine Scientific, Santa Ana, CA) that was supplemented with 0.2% human serum albumin (Irvine). The spermatozoa were washed twice with human tubal fluid–human serum albumin and were processed by centrifuge for 10 minutes at 300 $\times$ g. After the second wash, the supernatant was removed, and fresh human tubal fluid–human serum albumin was layered over the pellet and incubated for 60 minutes at 37°C. To retrieve the highly motile fraction, the volume from the top was removed. After the separation, the purified sperm population with high motility was resuspended in human tubal fluid–human serum albumin at a concentration of 5–10 $\times$ 10^6 spermatozoa per milliliter and was stored at –196°C without cryoprotectant until examined.

Samples were thawed in a 37°C water bath for 3 minutes immediately before assessment of DNA fragmentation and sperm shape (fixed wet preparation). An aliquot of approximately 25 μL was transferred to a multiwell slide (Cel-Line/Erie, Scientific Co, Portsmouth, NH) for examination of DNA fragmentation and morphological normalcy. Fragmentation of DNA and morphology were evaluated simultaneously in the same droplet and on the same sperm cell by using immunofluorescence and phase contrast, respectively. Each sample was analyzed in duplicate droplets, and the results were averaged.

Terminal Deoxynucleotidyl Transferase–Mediated Deoxyuridine Triphosphate–Fluorescein Nick-End Labeling Assay

Sperm DNA fragmentation was evaluated by TUNEL assay, using the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany). The assay uses fluorescein-dUTP to label single and double DNA strand breaks. It was performed according to the manufacturer's instructions and as published elsewhere (13). Each sperm suspension was aliquoted in 25-μL drops on a multiwell slide, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. This was followed by incubation in the dark at 37°C for 1 hour in TUNEL reaction mixture containing 0.5 IU/μL of calf thymus terminal deoxynucleotidyl transferase and fluorescein-dUTP. Negative (omitting the enzyme terminal transferase) and positive (using deoxyribonuclease I, 1 U/mL for 20 minutes at room temperature) controls were performed in each experiment. Mounting Medium for Fluorescence (Vectashield; Vector Laboratories, Burlingame, CA) was added, before the evaluation, to protect the fluorescence. A total of 200 cells (100 per well) was randomly analyzed per sample, by using a Nikon Eclipse E600 (Nikon, Tokyo, Japan) microscope at ×1,000 oil-immersion objective. Each sperm cell was diagnosed as having DNA intact (no fluorescence) or as having DNA fragmentation (intense green nuclear fluorescence).

Simultaneous Examination of Normal Sperm Morphology and DNA Fragmentation

Immediately after TUNEL, and in the same droplets used for DNA fragmentation analysis, sperm morphology (fixed wet preparation without staining) was assessed in several randomly selected fields under phase-contrast microscopy using a Nikon Eclipse E600 equipped with a SPOT-RT Slider digital camera (Diagnostic Instruments, Inc., Sterling, MI) using a ×1,000 oil-immersion objective. A total of 400 cells were evaluated, in two droplets per patient. During this examination, every time a spermatozoon with normal morphology was found, the light immediately was switched to the fluorescence to determine DNA integrity. Spermatozoa were considered normal when the head had a normal shape, a symmetrical and oval head configuration, vacuoles occupying <20% of the head area, an acrosomal region comprising 40%–70% of the head area, a symmetrical insertion of the tail, and absence of midpiece or neck defects (25–27).

Statistical Analysis

Data are expressed as mean ± SD. Comparisons were performed by using the Kruskal-Wallis test. The Mann-Whitney test was used to identify significant differences between groups. $P<.05$ was considered statistically significant.

RESULTS

The characteristics of the ejaculates of the groups analyzed are summarized in Table 1. Sperm concentration was 41.9 × 10^6/mL ± 18.4 × 10^6/mL (range, 23.4–60.3 × 10^6/mL) in the FER group, 52.5 × 10^6/mL ± 28.2 × 10^6/mL (range, 37.0–99.0 × 10^6/mL) in the SF group, and 92.0 × 10^6/mL ± 62.0 × 10^6/mL (range, 58.0–136.0 × 10^6/mL) in the INF group. The progressive motility was 64.2% ± 5.9% (range, 56.0–70.0%) for the FER group, 57.2% ± 21.5% (range, 24.0–77.0%) for the SF group, and 61.4% ± 16.8% (range, 41.4–95.1%) for the INF group. Results of morphology evaluation of the three groups stained with STAT III were as follows: 11.1% ± 0.6% (range, 10.5–12%) in the FER group, 4.3% ± 1.8% (range, 3.0–7.5%) in the SF group, and 3.8% ± 1.6% (range, 1.5–7.0%) in the INF group. The morphological abnormalities in all cases were predominantly in the head of the spermatozoa (severely amorphous heads), with occasional presence of vacuoles, and midpiece defects represented by cytoplasmic droplets; tail defects were present only in <5% of spermatozoa examined. There was no significant difference in sperm concentration ($P>.05$) and progressive motility ($P>.05$) between groups. The sperm morphology in the FER group was significantly higher

TABLE 1

Values of sperm concentration, motility and morphology in the semen samples.

Patient	Concentration (×I0^6/mL)[a]	Motility (%)[b]	Morphology (%)[c]
FER1	29.0	56.0	10.5
FER2	23.4	66.0	12.0
FER3	55.1	65.0	11.0
FER4	60.3	70.0	11.0
SF1	56.0	51.0	4.0
SF2	37.0	59.0	3.5
SF3	99.0	77.0	7.5
SF4	44.5	75.0	3.0
SF5	26.0	24.0	3.5
INF1	82.0	95.1	3.5
INF2	71.0	63.4	5.0
INF3	58.0	41.4	2.0
INF4	136.0	60.0	7.0
INF5	97.5	59.0	4.5
INF6	4.0	63.0	1.5
INF7	32.0	47.0	3.0
INF8	201.5	52.0	3.0
INF9	174.5	49.0	4.0
INF10	64.0	84.4	5.0

Note: Results of sperm concentration, motility and morphology in the studied ejaculates of the fertile (FER), subfertile (SF), and infertile (INF) men.

[a] $P>.05$ comparing the three groups regarding sperm concentration.

[b] $P>.05$ comparing the three groups regarding sperm motility.

[c] $P<.05$ comparing FER versus SF and FER versus INF.

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($P<.05$) than that in the SF and INF groups; there was no significant difference between SF and INF groups ($P>.05$).

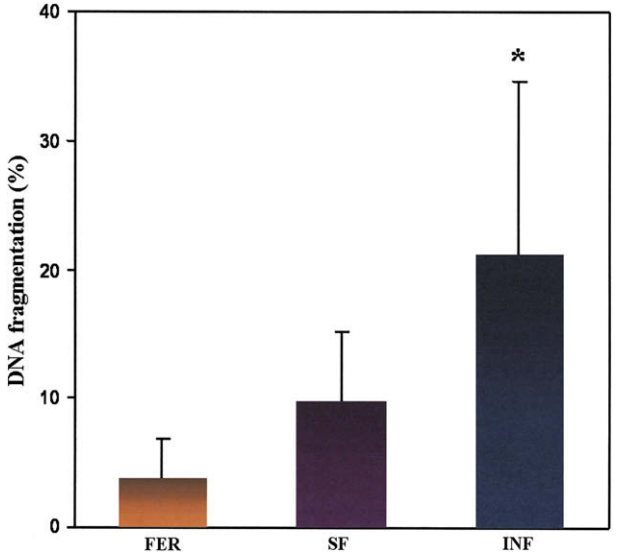
Motile spermatozoa selected by swim-up were used to perform TUNEL assay under fluorescence field. Then a simultaneous assessment of normal sperm morphology and DNA fragmentation was performed under phase-contrast microscopy, switched to fluorescence, using the ×1,000 oil immersion objective.

The proportion of TUNEL-positive cells was 3.9% ± 2.9% (mean ± SD) for the FER group, 9.8% ± 5.5% for the SF group, and 21.2% ± 13.4% for the INF group. There was a statistically significant difference between the FER and INF groups ($P<.05$). These results are presented in Figure 1.

The percentages of normal sperm morphology in the fixed-wet preparations without staining, as examined under phase contrast, were as follows: FER group, 7.5% ± 0.6%; SF group, 1.3% ± 1.7%; and INF group, 1.0% ± 0.3% ($P<.05$, FER vs. INF). Next, the spermatozoa with normal morphology were examined with fluorescence for TUNEL analysis. Representative photomicrographs are shown in Figures 2 and 3. No DNA fragmentation was found in spermatozoa with normal morphology in any of the samples from the FER group. In only one sample from the SF group did we observed normal spermatozoa exhibiting fluorescence (TUNEL positive). However, in all the samples from the patients in the INF group, we observed spermatozoa with normal morphology and with DNA fragmentation, with a high proportion of TUNEL-positive cells in all these subjects ($P<.05$ in INF vs. FER). Results are summarized in Table 2.

FIGURE 1

Incidence of spermatozoa with DNA fragmentation (TUNEL positive) in the fertile (FER), subfertile (SF) and infertile (INF) groups. A significant difference was found between FER and INF groups (*$P<.05$).



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DISCUSSION

To the best of our knowledge, this is the first study to simultaneously examine individual spermatozoa for normal morphology and DNA fragmentation. We demonstrate for the first time the presence of DNA fragmentation in spermatozoa with apparently normal morphology by using light microscopy. We examined the presence of DNA fragmentation in the elite population of swim-up–separated spermatozoa. The results demonstrated absent DNA fragmentation in normal spermatozoa from fertile men and demonstrated significantly higher levels in infertile men with moderate or severe teratozoospermia.

Further studies are needed to determine the presence of DNA fragmentation in infertile men with other sperm abnormalities, including various degrees of oligozoospermia. It is known that swim-up separation increases the population of highly motile and morphologically normal sperm (24). However, we did observe some discrepancy in the percentage of normal morphology in the raw (postliquefied) sample in air-dried and stained smears, compared with the results of the fixed-wet (unstained preparation) sample after swim-up and after TUNEL. This discrepancy can be explained by the fact that the recommended clinical procedures for evaluating human sperm morphology (air-dried and stained) can cause morphological artifacts and may not demonstrate small nuclear vacuoles (26–29). In addition, Meschede et al. (30) showed a significant difference between Papanicolaou stain (normal morphology: 31%) and wet preparations (12%). However, Oral and colleagues (31) compared Diff-Quik–stained and wet preparations for sperm morphology and did not find differences between the two methodologies.

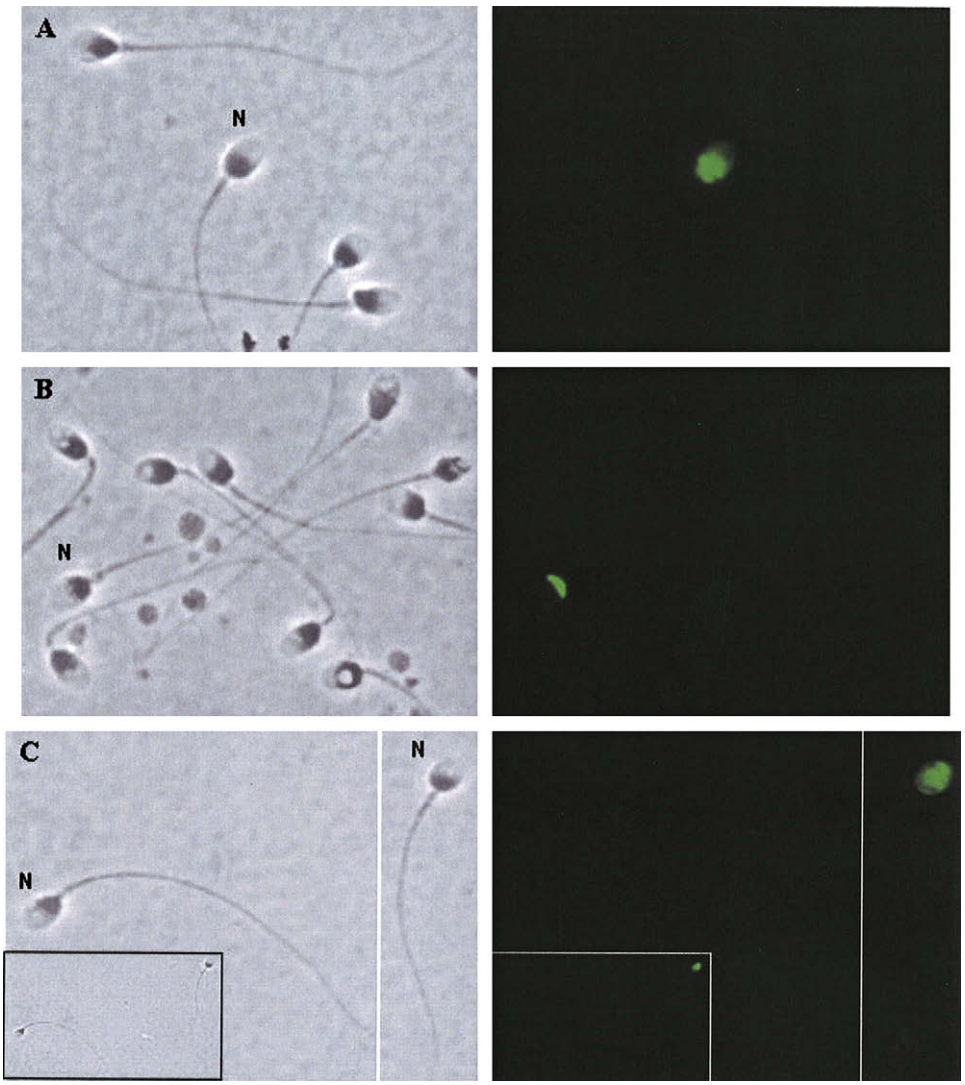
Intracytoplasmic sperm injection is used primarily for the treatment of infertile men with very poor sperm quality, and therefore a main concern is the possible inadvertent use of DNA-damaged spermatozoa to fertilize the oocyte. Reports have suggested potential adverse consequences such as fertilization failure, early embryo death, spontaneous abortion, childhood cancer, and infertility in the offspring (17).

Previous observations that sperm DNA damage is common in infertile men, together with the concerning preliminary reports on genetic and epigenetic abnormalities in children conceived through ICSI, urged us to explore the subject of sperm DNA damage further. Deoxyribonucleic acid that possesses measurable damage (for example, DNA fragmentation) may cause misreading errors to occur during DNA replication, and this may cause de novo mutations (32). Recent data point to the fact that the integrity of the paternal genome may have a critical role in human reproductive potential. The impact of an altered paternal genome on conception may be as detrimental as the impact of an altered maternal one. However, the true effect of DNA fragmentation of mature male germ cells on conception is still largely unknown.

Several studies have shown increased levels of sperm nuclear DNA damage in infertile men with abnormal sperm parameters (concentration, motility, and morphology) (12, 17, 33). Moreover, Saleh et al. (34) showed that men with normal basic sperm parameters may have significant levels of DNA

FIGURE 2

Representative photomicrographs of the simultaneous assessment of normal sperm morphology and DNA fragmentation following swim up separation (left side: phase contrast; right side: TUNEL fluorescence). **A:** a normal spermatozoon (N) with DNA fragmentation; note three morphologically abnormal spermatozoa without DNA fragmentation; **B:** a normal spermatozoon (N) with DNA fragmentation; note several amorphous spermatozoa without DNA fragmentation; **C:** two normal spermatozoa (N), one of them with DNA fragmentation. Inset: photomicrograph at lower magnification showing the two normal spermatozoa in the same field.



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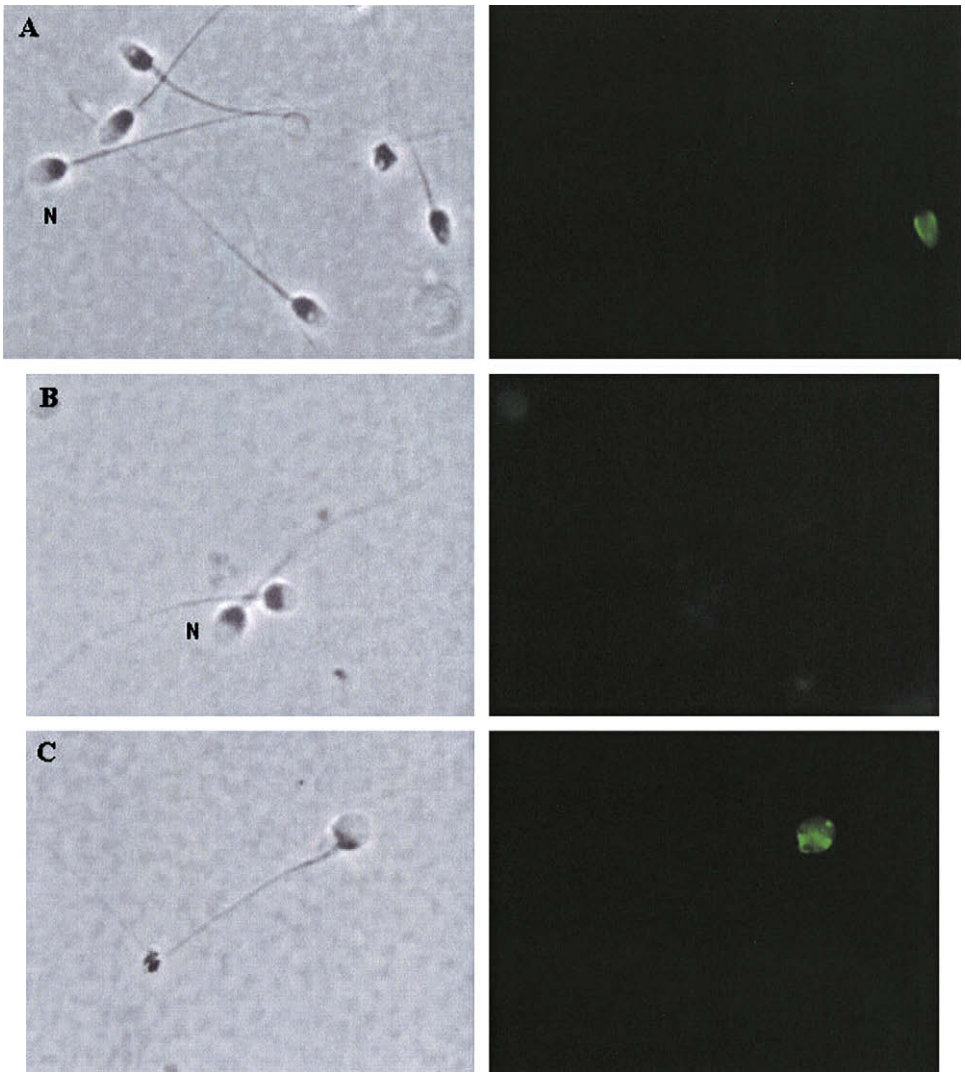
damage. Celik-Ozanci et al. (18) used phase-contrast microscopy and fluorescence in situ hybridization with centromeric probes for chromosomes X, Y, 10, 11, and 17 to evaluate human sperm shape and chromosomal aberrations in the same sperm cell of 15 men who presented for semen analysis. They found that sperm with normal shape could have chromosomal aberrations and concluded that sperm dimensions or shape are not reliable attributes in selection of haploid sperm for ICSI.

Burrello et al. (19) evaluated 10 patients with oligoasthenoteratozoospermia and 6 age-matched normozoospermic men. Those investigators analyzed morphology by using the same criteria recommended by the World Health Organization (27), but without Papanicolaou staining. The location of each spermatozoon was recorded by using an electronic microstage locator. Slides then were subjected to fluorescence in situ hybridization for chromosomes X, Y, 12. This study concluded that morphologically normal spermatozoa of oligoasthenoteratozoospermic patients carry an abnormal chromosomal constitution with the same frequency as that found in spermatozoa with an abnormal head shape.

By using high magnification for the morphological selection of motile spermatozoa, Berkovitz et al. (29) investigated

FIGURE 3

Representative photomicrographs of the simultaneous assessment of normal sperm morphology and DNA fragmentation following swim up separation (left side: phase contrast; right side: TUNEL fluorescence). **A:** a normal spermatozoon (N) without DNA fragmentation; note several abnormal spermatozoa, one of them with DNA fragmentation; **B:** a normal (N) and an abnormal spermatozoa without DNA fragmentation; and **C:** an abnormal spermatozoon with DNA fragmentation.



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the presence of large nuclear vacuoles and showed that ejaculates of men routinely referred for ICSI exhibited 30%–40% of spermatozoa with vacuolated nucleus. According to those investigators, in cases of spermatozoa with normal nuclear shapes but large vacuoles, the fertilization, embryonic cleavage, and implantation rates were normal; but the embryo developmental capacity in the later stages was compromised, as demonstrated by low pregnancy and high miscarriage rates. These results were supported by those of Thundathil et al. (35), who found that nuclear vacuoles in bovine sperm do not decrease fertilization rate but increase the rate of early embryonic death.

Tesarik et al. (20) analyzed the relationship between ICSI failure and sperm DNA fragmentation. Those investigators evaluated 18 infertile couples who had previously failed attempts of assisted reproductive techniques. In 8 couples, the adverse paternal effect did not produce any perceptible deterioration of zygote morphology. However, a late paternal effect was associated with an increased percentage of spermatozoa with fragmented DNA. Tesarik et al. (20) concluded that evaluation of sperm DNA integrity is useful for detecting late paternal effect, which is not associated with morphological abnormalities at the zygote and early cleavage stages.

TABLE 2

Swim up fractions: percentage of sperm with DNA fragmentation, percentage of sperm with normal morphology, and the proportion of normal sperm with DNA fragmentation.

Patient	TUNEL (%)[a]	Normal morphology (%)[b]	Normal sperm with positive TUNEL (%)[c]
FER1	0.5 (1/200)	7.00 (28/400)	0.0 (0/28)
FER2	6.5 (13/200)	8.00 (32/400)	0.0 (0/32)
FER3	6.0 (12/200)	8.00 (32/400)	0.0 (0/32)
FER4	2.5 (5/200)	7.00 (28/400)	0.0 (0/28)
SF1	4.0 (8/200)	1.00 (4/400)	0.0 (0/4)
SF2	5.0 (10/200)	1.25 (5/400)	0.0 (0/5)
SF3	12.5 (25/200)	4.25 (17/400)	47.0 (8/17)
SF4	17.5 (35/200)	0.25 (1/400)	0.0 (0/1)
SF5	10.0 (20/200)	0.00 (0/400)	0.0 (0/0)
INF1	5.0 (10/200)	1.00 (4/400)	50 (2/4)
INF2	23.0 (46/200)	1.25 (5/400)	60.0 (3/5)
INF3	5.5 (11/200)	0.75 (3/400)	33.3 (1/3)
INF4	27.0 (44/200)	1.50 (6/400)	50.0 (3/6)
INF5	26.0 (52/200)	0.75 (3/400)	66.6 (2/3)
INF6	38.5 (77/200)	0.5 (2/400)	50 (1/2)
INF7	39.5 (79/200)	1.75 (7/400)	42.9 (3/7)
INF8	31 (62/200)	1 (4/400)	25 (1/4)
INF9	10.5 (21/200)	1.25 (5/400)	40 (2/5)
INF10	6.5 (13/200)	1.25 (5/400)	20 (1/5)

Note: Results obtained from the swim up fractions of the three groups of patients. Results are expressed as: % TUNEL (number of TUNEL positive sperm cells/200 spermatozoa), % normal sperm morphology (number of normal sperm/400 spermatozoa), and the proportion of sperm with normal morphology that were TUNEL positive, calculated as the number of morphologically normal sperm being TUNEL positive/total number of spermatozoa with normal morphology.

[a] $P<.05$ FER versus INF.

[b] $P<05$ FER versus SF and FER versus INF.

[c] $P<.05$ FER versus SF and FER versus INF.

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Data from some publications have shown no relationship between sperm DNA damage and fertilization rates in ICSI (36). However, patients with low DNA fragmentation had a statistically significantly higher clinical pregnancy rate. These results may be accounted for by the fact that high DNA fragmentation does not preclude fertilization but may prevent blastocyst formation and/or successful embryo development (20, 21).

However, other investigators have concluded that DNA damage in spermatozoa is associated with reduced rate of IVF, impaired preimplantation development of the embryo, increased rates of early pregnancy loss, and poor fertility after natural or assisted conception (17). A number of theories have been advanced to explain the occurrence of sperm DNA fragmentation, including abortive apoptosis, oxidative stress associated with male genital tract infection, and defects of spermiogenesis (17, 37). The causes of sperm DNA damage appear to be multifactorial, and a solid conclusion about pathogenic mechanisms cannot yet be drawn.

It therefore is probable that the DNA damage present in a fertilizing spermatozoon will be transferred to the embryo. The fact that this damage may or not result in a given phenotype in the immediate offspring does not necessary mean, however, that the genetic disorders have not been transmitted to the progeny or that they will not become evident in future generations.

Under normal conditions, it has been speculated that the sperm DNA damage brought into the zygote effectively may be repaired by the oocyte. Shimura et al. (38) demonstrated the presence of a p53-dependent S-phase DNA damage checkpoint that could suppress DNA synthesis in both the male and female pronuclei before repair of the DNA damage. Nevertheless, it also is possible that the DNA repair capacity of the oocyte may be defective because of a high degree of DNA damage in the spermatozoon, or because of defects in the repair mechanisms themselves, as a consequence of factors such as maternal aging or in vitro culture conditions (39). In these situations, the damaged DNA could either remain unrepaired or be incorrectly repaired, leading to DNA mutations.

We conclude that [1] this is the first study to demonstrate that morphologically normal spermatozoa present in the motile sperm fractions of infertile men with moderate and severe

teratozoospermia can have DNA fragmentation; [2] further investigations and larger sample sizes are necessary to evaluate the real impact of DNA fragmentation in pregnancy outcome in patients undergoing ICSI; [3] the results pose a question about the use of so-called normal morphology alone as a reliable attribute for the selection of sperm for ICSI in this group of patients; and [4] methods that allow for an accurate separation of viable sperm with intact DNA should be sought for optimizing ICSI outcome. To further improve pregnancy rates and to prevent early childhood disease, more research is necessary to investigate these important findings.

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MODERN TRENDS

Edward E. Wallach, M.D.
Associate Editor

Developmental sperm contributions: fertilization and beyond

Gerardo Barroso, M.D., M.Sc.,[a] *Carlos Valdespin, M.D.,*[a] *Eva Vega, M.Sc.,*[a] *Ruben Kershenovich, M.D.,*[a] *Rosaura Avila, B.Sc.,*[a] *Conrado Avendaño, M.D.,*[b] *and Sergio Oehninger, M.D., Ph.D.*[b]

[a] Reproductive Medicine and Infertility Division, National Institute of Perinatology, Mexico City, Mexico; and [b] Obstetrics and Gynecology Department, Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, Norfolk, Virginia

The objective of this review was to examine the role of the various spermatozoal components suspected of actively participating in early human development. The contributions of the fertilizing spermatozoon to the oocyte include, as a minimum, the delivery of the DNA/chromatin, a putative oocyte-activating factor (OAF), and a centriole. Recent data indicate that spermatozoa may also provide the zygote with a unique suite of paternal mRNAs; some transcripts might be crucial for early and late embryonic development and deficient delivery, or aberrant transcription might contribute to abnormal development and arrest. Clinical evidence from assisted reproduction suggests that failure to complete the fertilization process, syngamy, or early cleavage might be the result of an early paternal effect. It is speculated that an abnormal release of a putative OAF and/or dysfunctions of the centrosome and cytoskeletal apparatus may mediate these effects. On the other hand, a later paternal effect resulting in embryonic failure to achieve implantation, pregnancy loss, and/or developmental abnormalities resulting from "carried over" sublethal effects may be associated with sperm nuclear/chromatin defects, including the presence of aneuploidy, genetic anomalies, DNA damage, and possibly other causes. These findings highlight the need for continuous monitoring of clinical results. (Fertil Steril® 2009;92:835–48. ©2009 by American Society for Reproductive Medicine.)

Key Words: Embryogenesis, fertilization, paternal contribution, spermatozoa

Past research has viewed the spermatozoon as a simple carrier or vector that transfers DNA to the egg, its contribution to embryogenesis being crucial for delivering the genetic material. It is now well established that there is extensive cross-talk between the fertilizing sperm and the egg, leading to activation of the egg on one hand and sperm head decondensation on the other. This is orderly followed by female and male pronuclear formation, syngamy, and the first cleavage divisions. Several structures/organelles and molecules present in the spermatozoon appear to be critical for the accomplishment of the milestones resulting in normal fertilization and early embryo development.

The fertilizing human spermatozoon is essential for contributing at least three components: [1] the paternal haploid genome, [2] the signal to initiate metabolic activation of the oocyte, and [3] the centriole, which directs microtubule assembly leading to the formation of the mitotic spindles during the initial zygote development. Clinical evidence derived from the use of assisted reproductive technology (ART) points to the fact that defective sperm contributions may extend beyond fertilization, highlighting the fact that early and late paternal effects may be determinants of abnormal development.

Intracytoplasmic sperm injection (ICSI) is a relatively new ART that involves injection of a mature spermatozoon into a metaphase II oocyte, typically performed in cases of male factor infertility. As such, this technique bypasses multiple steps of the natural fertilization process by introducing an apparently intact spermatozoon into the ooplasm. The impact of the microinjection technique on fertilization and postfertilization events should be definitely established to determine the immediate safety of ART as well as any possible long-term consequences, including embryonic anomalies that result in developmental arrest or are carried over to the offspring.

The objective of this review was to examine the role of the various spermatozoal components/molecules anticipated to participate in early human development. To accomplish this goal, we first summarize recent knowledge on the biology of sperm-egg interaction, focusing on data derived from both human and nonhuman models, and then discuss clinical evidence derived from the ART setting supportive of crucial contributions of the male gamete during early and late embryo development.

BIOLOGICAL CONSIDERATIONS

Sperm-Oocyte Interaction

It is generally accepted that to fertilize the egg, ejaculated spermatozoa must undergo capacitation, recognize and bind to the zona pellucida (ZP), and undergo the acrosome reaction (AR; Figs. 1 and 2)

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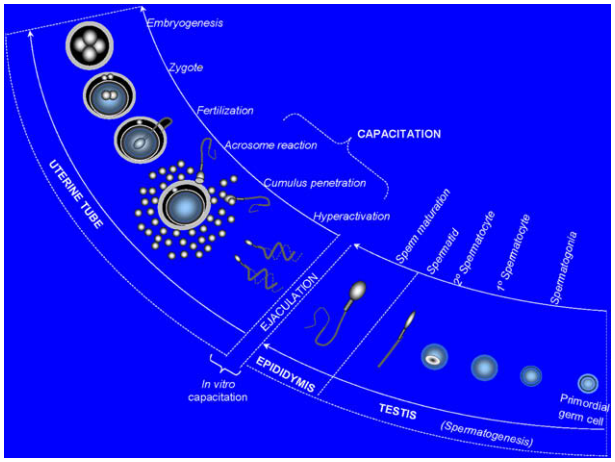
Reprint requests: Gerardo Barroso, M.D., M.Sc., Reproductive Medicine and Infertility Division, National Institute of Perinatology, Montes Urales 800, Col. Lomas de Virreyes, C.P. 11000; Mexico City, Mexico (FAX: 5255-55206238; E-mail: asistida@servidor.inper.edu.mx).

FIGURE 1

Schematic of the journey of the sperm cell during spermatogenesis and transit through the male and female tracts (modified from reference 1, with permission).



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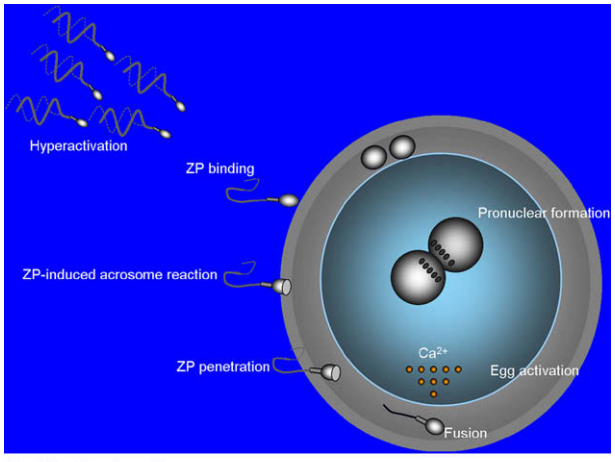
(1). The most significant changes experienced by sperm during capacitation are plasma membrane changes, increase in certain intracellular messengers, and increased phosphorylation of a set of proteins by different kinases (2–4).

In the murine species, a well-characterized model of gamete interaction, tight gamete binding is probably achieved through interaction of the ZP protein 3 (ZP3) and yet unidentified complementary sperm-binding protein(s) present in the plasma membrane. It is agreed that ZP3 triggers the AR that is then followed by a secondary binding process involving the ZP protein-2 (ZP2) and the inner acrosomal sperm membrane leading to zona penetration. Glycosylation appears mandatory for murine ZP3-ligand function. It has been demonstrated that O-glycosylation, and particularly terminal galactose residues of O-linked oligosaccharides, are essential for maintaining mouse gamete interaction. Others have provided evidence that the amino sugar N-acetylglucosamine is the key terminal monosaccharide involved in sperm-zona interaction in the mouse. In contrast, AR-triggering activity of ZP3 seems to depend upon the integrity of the protein backbone (5–7).

FIGURE 2

Critical steps involved in sperm-oocyte interaction leading to pronuclear formation and syngamy.



Note: ZP = Zona Pellucida.

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For the last two decades, investigators have sought to identify an individual protein or carbohydrate side chain as the "sperm receptor." In earlier work performed in nonhuman models, diverse candidates were postulated as primary sperm receptors for ZP3: [1] a 95 kd tyrosine-kinase (8); [2] sp56 (9); [3] trypsine-like protein (10); [4] β1-4 galactosyltransferase (11); and [5] spermadhesins (12). However, none of these molecules has been unequivocally established as an active receptor, and the physiological relevance of these candidates is still under debate.

In more recent experiments performed in knockout mice with absence of either ZP2 or ZP3 expression, it was demonstrated that the ZP fails to assemble around growing oocytes and that females are infertile; on the other hand, in the absence of ZP1 expression, a disorganized zona assembles around growing oocytes and females exhibit reduced fertility. These and other observations led to the speculation of a model for ZP structure in which ZP2 and ZP3 form long Z-filaments cross-linked by ZP1 (13).

The identity of the sperm-binding proteins remains a subject of active investigation, and the most recent data indicate that many different proteins are likely involved. There has been recent progress in functionally characterizing two promising candidate proteins that interact with ZP3: zonadhesin and PKDREJ; further, evolutionary analysis of both proteins within and between species of primates has identified promising regions of the proteins that may interact and coevolve with ZP3 (14–16).

In recently published studies, a group of investigators isolated, purified, and solubilized plasma membranes of porcine sperm heads (17). Three persistently bound proteins were identified by tandem mass spectrometry as isoforms of AQN-3, probably representing the main sperm protein involved in ZP binding in this species. This protein is a member of the spermadhesin family, a group of secretory proteins expressed in the epididymis. P47, fertilin beta, and peroxiredoxin 5 were also conclusively identified. More studies are needed to validate and characterize these proteins in other species.

Previous reports suggested that murine ZP2 mediates secondary binding of spermatozoa and that cleavage of ZP2 by proteases released through cortical granule reaction causes zona "hardening" and thus prevents polyspermy. Using an elegant approach, an observed postfertilization persistence of mouse sperm binding to "humanized" ZP was shown to correlate with uncleaved ZP2. These observations are consistent with a model for sperm binding in which the supramolecular structure of the ZP necessary for sperm binding is modulated by the cleavage status of ZP2 (18–21). To test the alternative hypothesis that the protein sequence of murine ZP glycoproteins mediates sperm-egg binding, transgenic mice were created in which mouse ZP3 was replaced with human ZP3. If initial gamete binding were protein mediated, such mice would bind human sperm. However, murine but not human sperm were found to bind to mouse eggs expressing human ZP3 (18).

During fertilization, the acrosome-reacted spermatozoa fuse with the oolema, and the whole spermatozoon (with the exception of part of the sperm membrane, most of the outer acrosomal membrane, and the acrosomal components) is incorporated into the oocyte (22). The

ZP binds to at least two different receptors in the sperm head plasma membrane. One is a G_i-coupled receptor that can activate phospolipase Cβ1 and regulates adenylyl cyclase to increase cyclic adenosine monophosphate (cAMP) levels. The cAMP activates protein kinase A (PKA) to open a calcium channel in the outer acrosomal membrane, resulting in a relatively small rise in cytosolic Ca^{2+}. Calcium activates phospholipase Cγ, which is coupled to the second tyrosine kinase receptor. The products of phospholipase C activity, diacylglycerol and inositol triphosphate (IP3), lead to activation of protein kinase C (PKC) and IP3 receptor; PKC opens a calcium channel in the membrane, and IP3 activates the calcium channel in the outer acrosomal membrane leading to a higher increase in cytosol calcium, which results in membrane fusion and completion of the AR (23–25). Results of recent studies indicate that components that are essential for intracellular membrane fusion in somatic cells, such as Rab3A, GTPase, and SNAREs, may be present in mammalian sperm and may also participate in membrane fusion during the AR (26).

A New Molecular Model for Human Sperm-Egg Interaction

Compelling evidence has now demonstrated that carbohydrate-binding proteins on the sperm surface mediate gamete recognition by binding with high affinity and specificity to complex glycoconjugates of the ZP (27–32). A group of investigators has recently reported a series of studies that suggest that [1] the binding protein(s) on human spermatozoa recognize selectin ligands or molecules alike on the ZP to ensure recognition and attachment; and [2] the human ZP expresses glycans structurally (and probably functionally) linked to natural killer cell inhibition (30, 32–36).

Those results are strengthened by the discovery that glycodelyn-A, an endometrial epithelial protein, produced a potent and dose-dependent inhibition of sperm-zona binding under in vitro conditions (32, 37, 38). This turned out not to be surprising since it was found that the oligosaccharides associated with this glycoprotein (fucosylated lacdiNAc antennae among others) also potently block selectin-mediated adhesions. Results also indicated the possibility that the carbohydrate binding specificity of the receptors mediating gamete recognition and lymphocyte/leukocyte adhesion have converged, at least to some extent. This concept is further supported by the demonstration of contraceptive and immunosuppressive properties of glycodelin-A and their carbohydrate dependence (30, 39, 40). Three major classes of N-glycans were detected, [1] high mannose, [2] biantennary bisecting type, and [3]) biantennary, triantennary, and tetra-antennary oligosaccharides terminated with $Lewis^x$ and $Lewis^y$ sequences (39–41). Thus, the major N-glycans of human sperm are associated with the inhibition of both innate and adaptive immune responses. These results provide more support for the eutherian "fetoembryonic defense system hypothesis" that links the expression of carbohydrate functional groups to the protection of gametes and the developing human in utero (30, 31).

Based on the previous data showing that glycodelin-A receptor(s) and ZP protein receptor(s) on human spermatozoa are closely related, Chiu et al. (42) used a chemical cross-linking approach to isolate the glycodelin-A sperm receptor complex from human spermatozoa. The receptor was identified to be fucosyltransferase-5 (FUT5) by mass spectrometry and confirmed with the use of anti-FUT5 antibodies. Biologically active FUT5 was purified from spermatozoa and bound strongly to intact and solubilized human ZP. These observations provided strong evidence that human sperm FUT5 is a receptor of glycodelin-A and ZP proteins and that glycodelin-A inhibits spermatozoa-zona binding by blocking the binding of sperm FUT5 to the ZP.

In the human, native solubilized ZP triggers the AR. Cross et al. (43) were the first to report that treatment of human sperm in suspension with acid-disaggregated human ZP (2–4 ZP/μL) increased the incidence of acrosome-reacted sperm. Lee, Check, and Kopf (44) demonstrated that pertussis toxin treatment of human sperm inhibits the (solubilized) ZP-induced AR. In contrast, acrosomal exocytosis induced by the calcium ionophore A-23187 is not inhibited by pertussis toxin pretreatment. Studies by Franken, Morales, and Habenicht (45) showed a dose-dependent effect of solubilized human ZP on the AR in the range of 0.25–1 ZP/μL and also confirmed the involvement of G_i protein during ZP-induced AR of human sperm. Schuffner and colleagues (46) reported that [1] acrosomal exocytosis of capacitated human sperm triggered by the homologous ZP is dependent on the activation of G(i) proteins (pertussis toxin sensitive) and the presence of extracellular calcium; and [2] P and follicular fluid exert a priming effect on the ZP-induced AR.

Recent studies have revealed that human ZP is comprised of four glycoproteins designated as human ZP1, ZP2, ZP3, and ZP4 (47, 48). The ortholog of the human *ZP4* gene is present in the mouse genome as a pseudogene. Chiu et al. (49) investigated the effects of native human ZP3 and ZP4 on AR and spermatozoa-ZP binding. Native human ZP3 and ZP4 were immunoaffinity purified. The investigators induced AR and inhibited spermatozoa-ZP binding time and dose dependently to different extents. These biological activities of human ZP3 and ZP4 depended partly on their glycosylation, with N-linked glycosylation contribution being more significant than O-linked glycosylation. Although progress is being made, the specific contributions of sperm-egg receptors/ligands involved in ZP binding are far from being resolved.

Sperm-Oocyte Fusion

Sperm-oocyte fusion is a cell-cell membrane fusion event. The inner and outer acrosomal membranes and the plasma membrane of the equatorial region remain intact after the completion of the AR and zona penetration (50, 51). Acrosome-reacted sperm bind to and fuse with the egg plasma membrane at the postacrosomal region of the sperm; this region is capable of fusion only after acrosomal exocytosis has taken place (28). Several candidate binding molecules have been reported.

Binding of sperm to the egg plasma membrane appears to be mediated by members of the cysteine-rich secretory protein family (CRISP1 and CRISP2), a member of the ADAM family of transmembrane proteins on sperm, and the integrin $alpha_{vi}ß_1$ receptor on eggs (52). Sperm binding to an egg integrin (ß1) is a prerequisite adhesion step for sperm-egg membrane fusion in mammalian fertilization (53). The oocyte integrin is required for membrane fusion, and its activity appears to be related to a sperm surface protein fertilin (termed PH-30) that was implicated in gamete fusion based on antibody inhibition studies. Although some ADAM proteins act to block or to promote protease activity, fertilin has no such roles (54).

P-selectin is expressed on the oolemma of human and hamster oocytes after sperm adhesion and is also detected on the equatorial region of acrosome-reacted human spermatozoa, suggesting that this selectin might be involved in gamete interaction (55). In addition, epididymal protein DE or CRISP1 and testicular protein Tpx-1 also known as CRISP2 are cysteine-rich secretory proteins that are also apparently involved in gamete fusion through interaction with egg-binding sites (56). Other candidates have been proposed, including equatorin and CD9 (57–59). Inoue et al. (60)

identified a mouse sperm fusion-related antigen and showed that the antigen belongs to a novel immunoglobulin superfamily protein. The investigators termed the gene Izumo and produced a gene-disrupted mouse line. Izumo2/2 mice were healthy, but males were sterile. They produced normal-looking sperm that bound to and penetrated the ZP but were incapable of fusing with eggs. Human sperm also contain Izumo, and addition of the antibody against human Izumo left the sperm unable to fuse with zona-free hamster eggs. However, glycosylation appears not to be essential for the function of Izumo (61). The specific roles of all these molecules need to be further validated (62).

Oocyte Activation

The signaling mechanism used by the spermatozoa to initiate and perpetuate oocyte responses is unclear, and three theories have been proposed: [1] the fusion theory, which suggests the presence of active calcium-releasing components in the sperm head (63, 64); [2] the receptor theory, which proposes a receptor-mediated signal transduction localized on the oocyte plasma membrane (65); and [3] the "calcium bomb" theory, which proposes that upon fertilization Ca^{2+} enters the egg either from stores in the sperm itself or through channels in the sperm's plasma membrane (66).

It was earlier reported that a cystosolic sperm factor containing a protein called oscillin, which is related to a prokaryote glucosamine phosphate deaminase and is located in the equatorial segment, appeared to be responsible for causing the calcium oscillations that trigger egg activation at fertilization in mammals (67). However, experimental evidence has now shown that oscillin is not responsible for the mammalian sperm calcium oscillations (68).

Present evidence supports the concept that an inositol-3-phosphate (IP3) receptor system is the main mediator of calcium oscillations in oocytes (67). It has recently been shown that the soluble sperm factor that triggers calcium oscillations and egg activation in mammals is a novel form of phospholipase C (PLC) referred to as PLC zeta (69). This has been demonstrated by injection into eggs of both c-RNA encoding for PLC zeta and recombinant PLC zeta (69, 70). According to a present hypothesis, after fusion of the sperm and egg plasma membrane, the sperm-derived PLC zeta protein diffuses into the egg cytoplasm, giving as a result the hydrolysis of PIP2 (phosphatidylinositol 4, 5-bisphosphate) from an unknown source to generate IP3 (inositol 1,4,5-trisphosphate) (71, 72).

The earliest visible indications of the transition of mammalian eggs, or egg activation, are cortical granule extrusion (CGE) by exocytosis and resumption of meiosis. Although these events are triggered by calcium oscillations, the pathways leading to the intracellular calcium release are not completely understood. The Ca^{2+} transients stimulate the resumption of the cell cycle by decreasing the activity of both an M-phase-promoting factor and a cytostatic factor (73) and either the Ca^{2+} transients and/or PKC lead to CGE (74). Therefore, the calcium transients and/or activation of PLC zeta lead to CGE by yet an undefined mechanism. Src family kinases (SFK) have been recently suggested as possibles inducers of some aspects of egg activation, although a role for SFK upstream of calcium release remains plausible (75).

Recently, two sperm-borne proteins that induce formation of pronuclei in eggs have been described: [1] the truncated c-Kit tyrosine kinase, which activates the dormant egg by eliciting intracellular Ca^{2+} oscillations, which serve as a secondary messenger for downstream effectors of zygotic development (76, 77); and [2] the protein PAWP (postacrosomal sheath WW domain binding protein). PAWP exclusively resides in the postacrosomal sheath of the sperm perinuclear theca (PT). Microinjection of recombinant PAWP or alkaline PT extract into metaphase-II-arrested porcine, bovine, macaque, and xenopus oocytes induced a high rate of pronuclear formation, which was prevented by coinjection of a competitive peptide derived from PAWP but not by coinjection of the point-mutated peptide. ICSI of porcine oocytes combined with coinjection of the competitive peptide or an antirecombinant PAWP antiserum prevented pronuclear formation and arrested fertilization (78).

Fate of Sperm Mitochondrial DNA

Most cells in the body contain between 103 and 104 copies of mitochondrial DNA (mtDNA). However, there are slightly higher copy numbers (n = 106) in mature oocytes. This may be in preparation for the energetic demands of embryogenesis (79–81). Spermatozoa, on the other hand, are metabolically flexible and, in some species, can switch between aerobic and anaerobic metabolism, which reflects the great range of oxygen tensions they experience, from near anoxia in the testis and epididymis, to ambient tension in the vagina environment and in vitro. Like somatic mitochondrial DNA (mtDNA), that of spermatozoa is highly vulnerable to mutation and a significant number of mtDNA deletions are found in the semen of at least 50% of normospermic men (82, 83).

Given the lengthy process of spermiogenesis and epididymal maturation during which the sperm and mitochondria have to survive, the likelihood of being exposed to mutagenic agents is high. Indeed, the need to exclude defective sperm mtDNA from contributing to the embryo is possibly one of the major selection pressures against survival of paternal mtDNA. Short (84) has suggested that the asymmetric inheritance of mtDNA may be the fundamental driving force behind amphimixis and anisogamy because of the need to conserve a healthy stock of mtDNA for embryo development through a long period of quiescence in meiosis.

The strictly maternal inheritance of mtDNA in mammals is a developmental paradox because the fertilizing spermatozoon introduces up to 100 functional mitochondria into the oocyte cytoplasm at fertilization (85). However, destruction of sperm mitochondria appears to be an evolutionary and developmental advantage (86) because the paternal mitochondria and their DNA may be compromised by the deleterious action of reactive oxygen species (ROS) encountered by the sperm during spermatogenesis, storage, migration, and fertilization (87). Studies have shown that the mitochondrial membrane proteins, rather than mtDNA, seem to determine whether the sperm mitochondria and mtDNA are passed on or degraded (88).

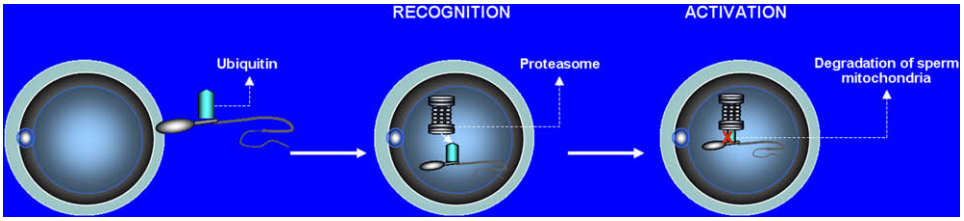
The Ubiquitin-Proteasome Pathway

Ubiquitin, a highly conserved protein present in apparently all eukaryotic cells, has the property of binding covalently to other proteins, via an isopeptide bond between the C-terminal glycine of ubiquitin and the E-amino group of a lysine in substrate proteins, a process called ubiquitination. The post-translational modification by ubiquitination marks defective or outlived intracellular proteins for proteolytic degradation by the 26S proteosome or by lysosomes (89). One of the most significant processes related to the ubiquitin-proteasome pathway in fertilization is the destruction of sperm mitochondria (90, 91) (Figure 3).

The true identity of ubiquinated substrates in the sperm mitochondria is not known. Nevertheless, it was recently shown that prohibitin, a mitochondrial membrane protein, is one of the ubiquitinated substrates that makes the sperm mitochondria responsible for the egg's ubiquitin-proteasome-dependent proteolytic machinery

FIGURE 3

Scheme of the ubiquitin-proteasome pathway in fertilization.



Barroso. Paternal contributions to the human embryo. Fertil Steril 2009.

after fertilization (92) Abnormalities of this recognition system might be involved in the deregulation of mitochondrial inheritance and sperm quality control.

Ubiquitin tagging of sperm mitochondrial membranes is not the only ubiquitin-proteasome pathway related to fertilization. During spermatogenesis, the ubiquitination process is indispensable in the replacement of the spermatid's nuclear histones by transition proteins, followed by permanent substitution with protamines (93, 94). Ubiquitination also has a principal role in the dramatic reduction of human sperm centrosome that occurs during spermatid elongation. Protein ubiquitination typically occurs in the cell cytosol or nucleus, and defective mammalian spermatozoa become ubiquitinated on their surface during post-testicular sperm maturation in the epididymus (95). Moreover, it has recently been postulated that sperm-acrosomal ubiquitin C-terminal hydrolases are involved in sperm-ZP interactions and antipolyspermy defense (96).

Morphologically normal and abnormal ubiquitinated sperm are found in the ejaculate of mammals (97, 98). The latter have apparently escaped from epididymal degradation and are characterized by main defects of head and/or axoneme (99). Sutovsky et al. (100, 101), found a discrete association of bovine semen ubiquitination with DNA fragmentation and suggested that sperm ubiquitination is associated with poor-quality sperm parameters in men. However, other studies have challenged this finding (102).

Pronuclear Interaction and Nuclear Fusion

In primates, the male pronucleus is tightly associated with the centrosome, which nucleates microtubules to form the sperm aster whose growth drives the centrosome and associated male pronucleus from the cell cortex toward the center of the oocyte. Structural abnormalities or incomplete junctioning of the centrosome have been identified as a novel form of infertility (103). In contrast, the female pronucleus has neither an associated centrosome nor microtubule nucleating activity. Nevertheless, the female pronucleus moves along microtubules from the cell cortex toward the centrosome located in the center of the sperm aster. The current model for the movement of the female pronucleus involves its translocation along the microtubule lattice using the minus-end-directed motor dynein in a manner analogous to organelle motility (104–106).

Mammalian fertilization requires dynein and dynactin to mediate genomic union; dynein concentrates exclusively around the female pronucleus, whereas dynactin localizes around the pronuclei and associates with nucleoporins and vimentin, in addition to dynein (107, 108). The findings that a sperm aster is required for dynein to localize to the female pronucleus and that the microtubules are necessary to retain dynein, but not dynactin, at their surface suggest that nucleoporins, vimentin, and dynactin might associate upon pronuclear formation and that subsequent sperm aster contact with the female pronuclear surface allows dynein to interact with these proteins (106–108).

EVIDENCE FOR PATERNAL CONTRIBUTIONS TO ABNORMAL FERTILIZATION AND EMBRYOGENESIS

Clinical Evidence: Lessons from the IVF/ICSI Setting

Successful fertilization is unequivocally dependent upon multiple inherent qualities of the oocyte (109, 110). In the last two decades, several lines of evidence resulting from the use of ART provided initial support for the concept of paternal contribution to faulty fertilization and abnormal embryogenesis. Strong evidence associated the presence of abnormal sperm parameters (particularly teratozoospermia, but also oxidative damage and DNA fragmentation) with failed or delayed fertilization and, importantly, to aberrant embryo development. These results are summarized in Table 1 (111–130).

The newly formed zygote undergoes early cleavage divisions depending upon the oocyte's endogenous machinery. Transcription is initiated at the 4- to 8-cell stage of the embryonic genome (131). Consequently, sperm nuclear deficiencies are usually not detected before the 8-cell stage, when a major expression of sperm-derived genes has begun. On the other hand, sperm cytoplasm deficiencies can be detected as early as the 1-cell zygote and then throughout the preimplantation development (127).

The terms "late" and "early" paternal effects have been proposed to denote these two pathological conditions (127, 132). The diagnosis of an early paternal effect is based upon poor zygote and early embryo morphology and low cleavage speed, and it is not associated with sperm DNA fragmentation. The late paternal effect, on the other hand, is manifested by poor developmental competence leading to failure of implantation and is associated with an increased incidence of sperm DNA fragmentation in the absence of zygote and early cleavage stage morphological abnormalities. It has been suggested that ICSI with testicular sperm can be an efficient treatment for the late paternal effect (133).

It can be speculated that the early paternal effect may include sperm dysfunctions related to oocyte activation (no sperm delivery of or dysfunctional oocyte-activating factor [OAF]) and aberrations of the centrosome-cytoskeletal apparatus. On the other hand, the late paternal effect is associated with sperm abnormalities at the level of DNA chromatin and perhaps sperm mitochondrial dysfunctions or abnormal sperm mRNAs delivery (see below). Alterations due to genomic imprinting anomalies probably result in both early and late paternal effects.

TABLE 1

Several lines of evidence resulting from the use of ART provide support for the concept of paternal contributions to faulty fertilization and abnormal embryogenesis.

Authors	Evidence
Kruger et al. 1988 (111); Oehninger et al. 1988 (112); Oehninger et al. 1988 (113); Oehninger et al. 1989 (114)	Abnormal sperm parameters, particularly teratozoospermia, are associated with fertilization disorders in IVF, including failure and delayed fertilization.
Ron-el et al. 1991 (115); Parinaud et al. 1993 (116)	Abnormal sperm parameters associated with embryo cleavage deficiencies.
Grow et al. 1994 (117); Oehninger et al. 1996 (118); Mercan et al. 1998 (119)	Although multiple studies have shown that the outcome of clinical pregnancies after ICSI is not affected by semen quality, patients with severe teratozoospermia demonstrated a low implantation rate.
Gorczyca et al. 1993 (120); Hughes et al. 1996 (121); Lopes et al. 1998 (122); Duran et al. 2002 (123); Liu et al. 2004 (124)	Spermatozoa from infertile subjects contain various nuclear alterations (abnormal chromatin structure, chromosomal abnormalities, microdeletions of Y chromosome, and DNA strand breaks).
Barroso et al. 2000 (125); Aitken et al. 2001 (126); Tesarik et al. 2004 (127); Barroso et al. 2006 (128)	Poor sperm quality is associated with increased sperm aneuploidy and DNA damage (fragmentation, instability, and single-stranded DNA).
Jakab et al. 2003 (129); Bartoov et al. 2005 (130)	Other sperm abnormalities have also been associated with failed fertilization and aberrant or arrested embryo development; studies suggest that abnormal sperm might be excluded using novel technologies.

Note: IVF = In vitro fertilization; ICSI = Intracytoplasmic sperm injection; DNA = Deoxyribonucleic acid.

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Disorders of Oocyte Activation, Centrosome-Cytoskeletal Apparatus Dysfunction, and Mitochondria Elimination

There are clinical situations that might be explained by absence or dysfunction of the OAF. For example, it has been suggested that up to 40% of failed fertilization cases after ICSI could be due to the failure of the egg to activate (134). In these cases, the sperm is within the cytoplasm but a stimulus for activation is apparently missing. Certainly, there could be other cases in which the sperm provides the OAF but any of the multiple elements of the oocyte responsive system (SFKs, PIP2, 1P3 receptor, or PKC) could be aberrant, resulting in failure to resume meiosis or to undergo CGE.

It has recently been reported that sperm from a group of patients who repeatedly failed ICSI had undetectable PLC zeta and were unable to induce $Ca2^{+}$ oscillations in mouse eggs (135). Using mouse eggs, the investigators rescued egg activation by injection of mouse PLC zeta mRNA, indicating that the inability of human sperm to initiate $Ca2^{+}$ oscillations lead to failure of egg activation. Such results provide strong evidence for an abnormal PLC zeta expression underlying this functional defect.

Dysfunctional microtubule organization in failed fertilization during human IVF suggested that centrosomal dysfunction might be a cause of fertilization arrest. Microtubules and DNA were imaged in inseminated human oocytes that had been discarded as unfertilized (136). Results showed that fertilization arrested at various levels: [1] metaphase II arrest, [2] arrest after the successful incorporation of the spermatozoon, [3] arrest after the formation of the sperm aster, [4] arrest during mitotic cell cycle progression, and [5] arrest during meiotic cell cycle progression. In another study, it was demonstrated by inmunofluorescence analysis that a main reason of fertilization failure after IVF was no sperm penetration (55.5%). On the other hand, fertilization failure after ICSI was mainly associated with incomplete oocyte activation (39.9%) (134).

In a nonhuman primate model using apparently normal gametes, ICSI resulted in abnormal nuclear remodeling during sperm decondensation due to the presence of the sperm acrosome and apical perinuclear theca, structures normally removed at the oolema during IVF; this in turn caused a delay of DNA synthesis (137). Such unusual modifications brought concerns about the normalcy of the fertilization process and cell cycle checkpoints during ICSI (138).

The sperm acrosome contains a variety of hydrolytic enzymes that release into the ooplasm and might generate some kind of damage (139). It is unclear how an oocyte that has been microinjected with an acrosome-intact spermatozoon will cope with the sperm acrosome. It is believed that an acrosome introduced into the ooplasm by ICSI seems to disturb sperm chromatin decondensation physically. Katayama, Koshida, and Miyake (140) showed detailed morphological characteristics of the acrosome of boar sperm through ICSI, showing that the RNA-binding properties of sperm head components introduced into the cytoplasm were different from those after IVF. Resumption of meiosis and cortical- granules exocytosis was achieved after micromanipulation techniques.

Terada et al. (141) assessed the centrosomal function of human sperm using heterologous ICSI with rabbit eggs. They demonstrated that the sperm aster formation rate was lower in infertile men compared with in controls. Moreover, the sperm aster formation rate correlated with the embryonic cleavage rate after human IVF. The data suggested that reproductive success during the first cell cycle requires a functional sperm centrosome; dysfunctions of this organelle could be present in cases of unexplained infertility.

Kovacic and Vlaisavljevic (142) studied the microtubules and chromosomes of human oocytes failing to fertilize after ICSI. The results showed a high proportion of oocytes arrested at metaphase II; it was concluded that sperm that do not activate the oocyte might continue decondensing the chromatin. However, the oocyte prevents

male pronucleus formation before the female one, mostly by causing premature chromatin condensation in the sperm and by duplicating the sperm centrosome.

The functional role of the sperm tail in early human embryonic growth is not known. In microinjection experiments, it was demonstrated that injection of isolated sperm segments (heads or flagella) could permit oocyte activation and bipronuclear formation. However, a high rate of mosaicism was observed in the embryos with disrupted sperm, suggesting that the structural integrity of the intact fertilizing spermatozoon appears to contribute to normal human embryogenesis (143). In addition, oocytes injected with mechanically dissected spermatozoa, although capable of pronuclear formation, did not undergo normal mitotic division. The lack of a bipolar spindle, in combination with mosaicism, suggested abnormalities of the mitotic apparatus when sperm integrity is impaired after dissection (144).

Occasional occurrence of paternal inheritance of mtDNA has been suggested in mammals, including humans (145). While most such evidence has been widely disputed, of particular concern is the documented heteroplasmic or mixed mtDNA inheritance after ooplasmic transfusion (146). Indeed, there is evidence that heteroplasmy is a direct consequence of ooplasm transfer, a technique that was used to "rescue" oocytes from older women by injecting ooplasm from young oocytes. ICSI has inherent potential for delaying the degradation of sperm mitochondria. However, paternal mtDNA inheritance after ICSI has not been documented.

Putative Dysfunctions Resulting from Aberrant Delivery of mRNAs

New evidence has challenged the traditional view of the transcriptional dormancy of terminally differentiated spermatozoa. Several reports indicated the presence of mRNAs in ejaculated human spermatozoa (147, 148). It has been hypothesized that these templates could be critically involved in late spermiogenesis, including a function to equilibrate imbalances in spermatozoal phenotypes brought about by meiotic recombination and segregation, but also that they could be involved in early postfertilization events such as establishing imprints during the transition from maternal to embryonic genes. Others have instead proposed that mature spermatozoa are a repository of information regarding meiotic and postmeiotic gene expression in the human and are likely to contain transcripts for genes playing an essential role during spermiogenesis. The use of the whole ejaculate as a wholly noninvasive biopsy of the spermatid should therefore be evaluated (149).

A nonexhaustive list of transcripts include c-myc, HLA class 1, protamines 1 and 2, heat shock proteins 70 and 90, ß-integrins, transition protein-I, beta-actin, variants of phosphodiesterase, P receptor, and aromatase, as well as an extended pattern of several transcripts encoding factors (NF_kB, HOX2A, ICSBP, JNK2, HBEGF, RXRβ, and ErbB3), revealing a wide range of transcripts in mammalian, including human, sperm. The presence of residual DNA and RNA polymerase activity within the sperm chromatin has also been formerly reported (150–154).

Complementary investigations have indicated that in spite of a high degree of DNA packaging within the human sperm head, chromatin retains some features of active chromatin, mainly acetylated histones, and the arrangement of certain chromatin domains into the nucleosomes (155–158). The existence of translational activities in human sperm during capacitation and AR has been described, which could also explain the presence of mRNA in mature sperm (159).

It is possible that if the mRNAs accumulated in the sperm nucleus are not residual nonfunctional materials, they might be viewed as the male gametes' contribution to early embryogenesis (160). Delivering spermatozoa RNA to the oocyte has been demonstrated in mice (161) and humans (147). Some sperm transcripts encoding proteins known to participate in fertilization and embryonic development have been specifically detected in early embryos after IVF failure, while they have not been found in the oocyte. Thus, human spermatozoa could act not only as genome carriers but also as providers of specific transcripts necessary for zygote viability and development before activation of the embryonic genome.

In this regard, Avendaño et al. (162) recently examined the possibility that some mRNA transcripts present in mature ejaculated human spermatozoa can survive after fertilization and might therefore play a function in the new cell. Two transcripts (PSG1 and HLA-E) with known roles in implantation were studied; these transcripts are human specific (absent in hamster eggs and present in human sperm). Results demonstrated that these messengers were present 24 hours after fertilization in a heterologous model (human sperm injected into hamster oocytes by ICSI), indicating that the mature spermatozoa deliver mRNA into the oocyte and that these molecules can selectively survive and possibly play a role in embryo development.

Aberrant Embryogenesis Secondary to Nuclear/Chromatin Anomalies

In human spermatozoa, 15%–20% of histones are retained in the nucleus to coexist with protamines. Hypothetically, nucleohistone regions of sperm chromatin mark DNA sequences for distinctive processing during fertilization and early embryogenesis. Nazarov et al. (163) have proposed a novel model for the nuclear architecture of human spermatozoa. Elaborate nonrandom organization of human sperm chromosomes at different structural levels, starting from the DNA packing by protamines up to the higher-order chromosome configuration and nuclear positioning of chromosome territories, has been discovered. Zalensky and Zalenskaya (164) have put forward a hypothesis that the unique genome architecture in sperm provides a mechanism for orchestrated unpacking and ordered activation of the male genome during fertilization, thus offering an additional level of epigenetic information that will be deciphered in the descendant cells.

During the process of mammalian spermiogenesis, a significant reorganization of the chromatin structure occurs involving the sequential substitution of somatic histones with protamines. In the human sperm nucleus, approximately 15% of the basic nuclear protein complement is maintained as histones. Human testis/sperm-specific histone H2B (hTSH2B) is a variant of the histone H2B expressed exclusively in spermatogenic germline cells and present in some mature sperm cells. Thus, this protein marks a subpopulation of sperm cells in the ejaculate (165, 166). Using indirect immunofluorescence, Singleton et al. (165) examined the influence of hTSH2B on ZP binding and sperm head decondensation in amphibian egg cell-free extract. As suggested by previous studies, they reported that hTSH2B can be localized in only approximately 30% of sperm cells within a given ejaculate. The investigators established that the presence of hTSH2B does not influence sperm ZP binding capacity. Finally, it was found that decondensation occurred more rapidly and to a greater extent in those cells containing hTSH2B. It is proposed that the presence or absence of hTSH2B within spermatozoa

influences pronuclei formation and the activation of paternal genes after fertilization and during early embryonic development.

Esterhuizen et al. (167) evaluated the role of chromatin packaging (CMA3 staining), sperm morphology during sperm-zona binding, sperm decondensation, and the presence of polar bodies in oocytes that failed IVF. Odds ratio analyses indicated that being in the $\geq$60% CMA3 staining group resulted in a 15.6-fold increase in the risk of decondensation failure, relative to CMA3, with staining of <44%. Therefore, sperm chromatin packaging quality and sperm morphology assessments are useful clinical indicators of human fertilization failure.

Ejaculated human spermatozoa may present various degrees of DNA damage. Different theories have been proposed to explain its origin (125, 168–171): [1] damage could occur at the time of or be the result of DNA packing during the transition of histone to protamine complex during spermiogenesis; [2] DNA fragmentation could also be the consequence of direct oxidative damage that has been associated with antioxidant depletion, smoking, xenobiotics, heat exposure, leukocyte contamination of semen, and presence of ions in sperm culture media; and [3] DNA damage could be the consequence of apoptosis.

The presence of apoptosis in ejaculated spermatozoa could be the result of various types of injuries (125, 170, 171). In vivo, apoptosis could be triggered at the testicular (hormonal depletion, irradiation, toxic agents, chemicals, and heat have been shown to induce apoptosis), epididymal (the result of signals released by abnormal and/or senescent spermatozoa or by leukocytes, such as

TABLE 2

Compelling evidence of the presence of somatic cell apoptotic markers in human ejaculated spermatozoa as reported by our laboratories.

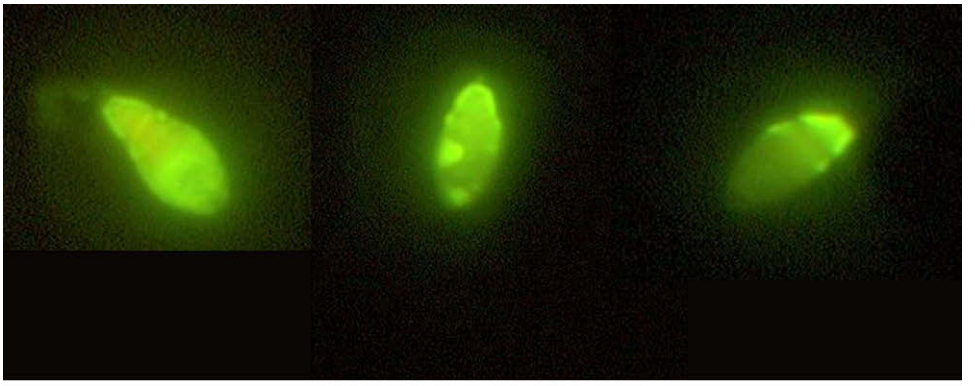
Authors	Evidence
Barroso et al. 2000 (125); Schuffner et al. 2002 (176); Weng et al. 2002 (177)	Early and late apoptotic markers were demonstrated using annexin V and TUNEL. Also, an important role of activated and nonactivated caspases was shown by using specific monoclonal antibodies.
Barroso et al. 2000 (125); Weng et al. 2002 (177)	The apoptotic markers: PS externalization and DNA fragmentation are expressed with a higher frequency in the fractions of sperm with low motility.
Weng et al. 2002 (177); Oehninger et al. 2003 (172)	Apoptotic markers are expressed with a significantly higher frequency in spermatozoa from infertile men when compared with fertile controls.
Weng et al. 2002 (177)	Caspase-3, the major executioner caspase, was demonstrated in human sperm in both active and inactive forms. Apparently, active caspase-3 was exclusively detected in the midpiece, where mitochondria and residual cytoplasm are present.
Taylor et al. 2004 (178)	Human sperm exhibit other members of the caspase family, caspase-7 and -9. By immunoblotting, we demonstrated the presence of inactive caspase-7 and caspase-9 in many samples, as well as active caspase-7 and caspase-9 in samples of infertile men.
Taylor et al. 2004 (178)	Human sperm possess the AIF. By immunoblotting, we have demonstrated that human sperm express AIF. Additionally, sperm expresses unique poly-ADP-ribose polymerase, a specific caspase substrate found in somatic cells.
Taylor et al. 2004 (178)	Caspase activation can be triggered in ejaculated human sperm by the mitochondrial disruptor staurosporine, which significantly enhanced caspase activation and DNA fragmentation.
Taylor et al. 2004 (178); Castro et al. 2004 (179)	Human sperm did not trigger or exhibit any response to Fas ligand in experiments testing caspase activation, PS translocation, or DNA fragmentation.
Duru et al. 2001 (180); Oehninger et al. 1995 (181)	Hydrogen peroxide (H_2O_2), the most damaging ROS in sperm, induces the expression of apoptotic markers by increasing PS translocation and DNA fragmentation.
Barroso et al. 2000 (125)	Ejaculated human sperm show a strong correlation between ROS production and DNA fragmentation, linking mitochondrial dysfunction and expression of apoptosis markers.
Barroso et al. 2006 (128)	Ejaculated sperm show a strong correlation between disruption of the mitochondrial potential membrane and PS translocation.

Note: AIF = apoptotic inducing-factor (AIF); PS = phosphatidylserine; TUNEL = TdT-mediated dUTP Nick-End Labeling; DNA = Deoxyribonucleic acid; ROS = Reactive oxygen species.

Barroso. Paternal contributions to the human embryo. Fertil Steril 2009.

FIGURE 4

Three different patterns of DNA fragmentation (TUNEL assay) frequently observed in infertile men (left: granular appearance throughout the sperm head; center: predominance of vacuolated appearance; right: homogeneous fluorescence throughout nuclear area).



Note: TUNEL = TdT-mediated dUTP Nick-End Labeling.

Barroso. Paternal contributions to the human embryo. Fertil Steril 2009.

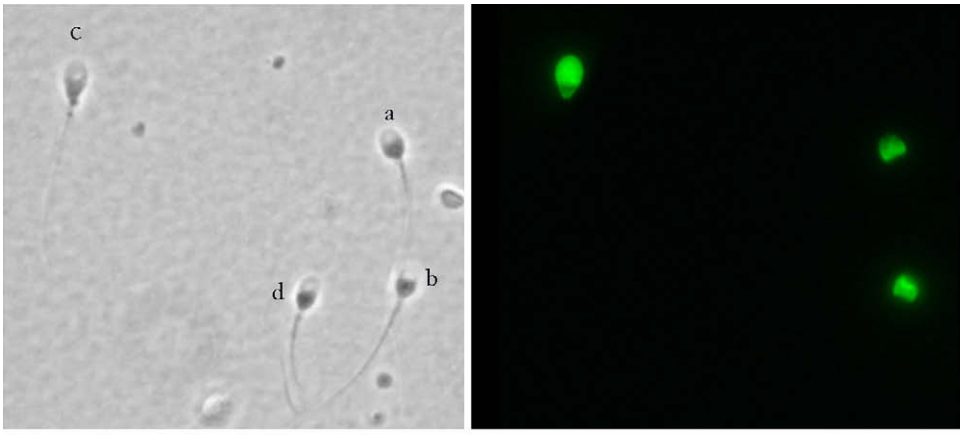
ROS and other mediators of inflammation/infection), or seminal (ROS, lack of antioxidants, or other causes) levels. In addition, apoptosis could be triggered by factors present in the female tract. In vitro, apoptosis could be triggered upon incubation with inappropriate culture media or other manipulation procedures. Irrespective of the stimulus, spermatozoa undergoing apoptosis and unrecognized by currently used methodologies may be dysfunctional. More dramatically, they may pose the risk of carrying a damaged genome into the egg, resulting in poor embryo development, miscarriage, or birth defects (170).

Compelling evidence indicates the presence of somatic cell apoptosis markers in human ejaculated spermatozoa (168–172). Evidence gathered in our laboratories is summarized in Table 2 (125, 128, 173–177). The oocyte has the capability to repair the DNA damage as oocytes fertilized by DNA-damaged spermatozoa did not develop further in vitro when they were cultured in the presence of inhibitors to DNA repair (178). But the capacity of the oocyte to repair is limited and is related to the degree of sperm DNA damage. The fertilization capacity of apoptotic sperm has been observed at the same rate as intact spermatozoa; however, the in vitro embryo development to the blastocyst stage is closely related to the integrity of the DNA (179).

It has been known for many years that the chromatin of the mature sperm nucleus can be abnormally packaged (180). In addition,

FIGURE 5

Morphologically normal sperm with fragmented DNA (normal-SFD) in the same semen sample of patients currently undergoing ICSI. Simultaneous assessment of normal sperm morphology (phase contrast, unstained) and DNA fragmentation (TUNEL) after swim-up separation (left side: phase contrast; right side: TUNEL fluorescence). (A) Normal spermatozoon with DNA fragmentation, (B) abnormal spermatozoon (slightly abnormal form) with DNA fragmentation, (C) morphologically abnormal spermatozoa (severely amorphous) with DNA fragmentation, and (D) morphologically abnormal spermatozoa (tapered form) without DNA fragmentation.



Note: DNA = Deoxyribonucleic acid; ICSI = Intracytoplasmic sperm injection; TUNEL = TdT-mediated dUTP Nick-End Labeling.

Barroso. Paternal contributions to the human embryo. Fertil Steril 2009.

abnormal chromatin packaging and nuclear DNA damage appear to be linked, and there is a strong association between the presence of nuclear DNA damage in the mature spermatozoa of men and poor semen parameters (170–172, 181). Endogenous nicks in DNA are normally expressed at specific stages of spermiogenesis in different animal models; these endogenous nicks are evident during spermiogenesis but are not observed once chromatin packaging is completed. It is postulated that an endogenous nuclease, topoisomerase 11, creates and ligates nicks to provide relief of torsional stress and to aid chromatin rearrangement during protamination. (182–186).

Several studies have shown that sperm DNA quality had robust power to predict fertilization in vitro (187–189). Tomlinson et al. (190) reported that the only parameter showing a significant difference between pregnant and nonpregnant groups in IVF was the percentages of DNA fragmentation assessed by in situ nick translation. The sperm chromatin structure assay (SCSA) has been proposed as a diagnostic tool to predict fertilization by evaluating sperm DNA stability (191). The SCSA measures susceptibility to DNA denaturation in situ in sperm exposed to acid for 30 seconds followed by acridine orange staining. The use of flow cytometry in the SCSA increases its dependability.

Duran et al. (123, 192), studied a large infertility population undergoing IUI in a prospective cohort fashion. A total of 119 patients underwent 154 cycles of IUI. DNA fragmentation evaluated by terminal deoxynuceotidyl transferase-mediated UTD nick-end labeling (TUNEL) and acridin orange staining was measured. The investigators reported that sperm DNA quality played a major role as a predictor of pregnancy under such in vivo conditions. Figure 4 shows three different patterns of DNA fragmentation frequently observed in infertile men. More studies are needed to determine the clinical significance of these finding in terms of fertilization and developmental potential.

Avendaño et al. (193) recently reported that infertile men can present DNA fragmentation in the morphologically normal spermatozoa recovered post-swim-up. In addition, using simultaneous evaluation of DNA integrity and sperm morphology in the same sperm cell (Figure 5), the same investigators evaluated morphologically normal sperm with fragmented DNA (normal-SFD) in a fraction of the same semen sample of patients currently undergoing ICSI. Findings demonstrated a significant and negative correlation between the percentage of normal-SFD and embryo quality and, more importantly, with pregannсy outcome (194).

Muratori et al. (195) identified a group of round bodies without a nucleus of different size and density in human semen. These bodies are positive for the fluorochrome merocyanine 540 staining after which they were named. It has been postulated that these elements are residues of apoptotic germ cells, demonstrated by the presence of some apoptotic markers (caspase activity, FAS, p53 and Bcl-x, and DNA fragmentation). Besides, it was also observed that these elements are especially frequent in oligoasthenoteratozoospermic patients. An apparently logical explanation that the investigators have developed is that these findings suggest that these apoptotic bodies have somehow escaped from testicular or epididymal phagocytosis.

Using flow cytometry, it is possible to distinguish sperm from M540 bodies by labeling samples with nuclear probes because the latter fail to stain M540 bodies. In two studies performed with flow cytometry to study sperm ubiquitination and DNA fragmentation in which M540 bodies were both included and excluded, it was demonstrated that M540 bodies largely affected the results (196). Future investigations should take into consideration the information reported by these studies to thoroughly analyze the degree of DNA fragmentation in sperm subpupulations (197).

CONCLUSIONS

This review highlighted the concept that the spermatozoon has a very dynamic and critical participation in normal embryogenesis that clearly extends beyond the fertilization process. Progress is slowly being made related to the unveiling of molecules involved in sperm-oocyte interaction and regulators of fertilization and post-fertilization developmental steps. Furthermore, we presented unequivocal clinical evidence that defective spermatozoa that penetrate the oocyte may cause arrest of development at multiple levels during embryo preimplantational development. Additional data suggest that sublethal effects can be "carried over" after implantation, resulting in untoward embryonic/fetal defects. These findings highlight the need for continuous monitoring of ART results.

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MALE FACTOR

DNA fragmentation of normal spermatozoa negatively impacts embryo quality and intracytoplasmic sperm injection outcome

Conrado Avendaño, M.S., Anahí Franchi, Ph.D., Hakan Duran, M.D., and Sergio Oehninger, M.D., Ph.D.

The Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, Virginia

Objective: To evaluate DNA fragmentation in morphologically normal sperm recovered from the same sample used for intracytoplasmic sperm injection (ICSI) and to correlate DNA damage with embryo quality and pregnancy outcome.
Design: Prospective study.
Setting: Academic center.
Patient(s): 36 infertile men participating in the ICSI program.
Intervention(s): Terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate-fluorescein nick end labeling (TUNEL) assay and morphologic assessment by phase contrast.
Main Outcome Measure(s): Simultaneous assessment of sperm morphology and DNA fragmentation by TUNEL assay was performed in the same cell, then the percentage of normal sperm with fragmented DNA (normal SFD) was correlated with embryo quality and pregnancy outcomes.
Result(s): A highly statistically significant negative correlation was found between the percentage of normal SFD and embryo quality. This association was confirmed for the transferred embryos and for the total embryo cohort. The receiver operating characteristics curve analysis demonstrated that the percentage of normal SFD and embryo quality were statistically significant predictors of pregnancy. When the percentage of normal SFD was $\leq$17.6 %, the likelihood of pregnancy was 3.5 times higher. No correlation was found between the percentage of total sperm with fragmented DNA (morphologically normal and abnormal) and ICSI outcomes.
Conclusion(s): The DNA fragmentation of morphologically normal sperm negatively impacts embryo quality and probability of pregnancy in ICSI cycles. (Fertil Steril® 2010;94:549–57. ©2010 by American Society for Reproductive Medicine.)

Key Words: Embryo quality, ICSI outcome, sperm DNA fragmentation, sperm morphology

Intracytoplasmic sperm injection (ICSI) has been recognized as a very efficient treatment modality for male factor infertility cases who present with poor sperm quality and/or failed conventional in vitro fertilization (IVF) cycles. The selection of the spermatozoon to be used for ICSI is based on the judgment of the embryologist, who chooses a motile spermatozoon with as good morphologic appearance as possible. However, the selected spermatozoa may have damaged DNA (1).

Many investigators (1–5) have shown "paternal effects" that can lead to a decrease in the success of assisted reproductive techniques (ART). A late paternal effect (2) has been mainly attributed to anomalies in the organization of the sperm chromatin (i.e., reduced chromatin condensation, chromosome anomalies, and increased DNA strand breaks or fragmentation). Several studies have suggested that paternal genomic alterations can compromise fertilization rate and embryo viability/quality and result in increased spontaneous miscarriage and minor or major birth defects (1, 3).

Sperm DNA fragmentation has been found to be associated with reduced natural conception, intrauterine insemination (IUI), and IVF outcome rates (6–13). For ICSI, contradictory results have been reported. While some reports did not find an effect (9, 14), others have shown a significant influence of sperm DNA fragmentation on fertilization and pregnancy rates (15–17). These discrepancies could be explained by the fact that during ICSI the sperm selection is based in the motility and the apparently normal morphology. However, the integrity of the DNA in the selected spermatozoon could be a major determinant of the overall success of this procedure.

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Reprint requests: Sergio Oehninger, M.D., Ph.D., The Jones Institute for Reproductive Medicine, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, 601 Colley Avenue, Norfolk, Virginia 23507 (FAX: 757-446-8998; E-mail: oehninsc@evms.edu).

It has been shown that sperm with fragmented DNA can fertilize eggs with the same efficiency as sperm without DNA fragmentation (18); however, if critical genes are damaged when the paternal genome is activated at day 3 (four to eight cell stage), embryo development failure is likely to occur. The inadvertent selection of spermatozoa with damaged DNA for ICSI may have untoward effects, compromising not only the normality of the embryos but also the resultant offspring. This highlights the need for strict monitoring and follow-up observation of the long-term health of children conceived by this technique. Nevertheless, there is sufficient evidence to suggest a negative effect of the use of spermatozoa with fragmented DNA (1). In addition, the negative consequences of using sperm with damaged DNA for short-term and long-term health have been recently demonstrated using animals models (19).

Based on the previous reports and our recent finding that motile spermatozoa with morphologically normal appearance can have damaged DNA (20), we further investigated the impact of DNA fragmentation in morphologically normal sperm on ICSI outcome, measured in terms of embryo quality and pregnancy potential. We focused our study on the identification of DNA fragmentation not only in the motile sperm (recovered by the swim-up technique) but also in the morphologically normal spermatozoa because these are the cells with a high probability of being selected by the embryologist at the time of oocyte injection for ICSI.

MATERIALS AND METHODS

Patients

This was a prospectively designed clinical study. The institutional review board of Eastern Virginia Medical School approved the study, and all participants gave written informed consent. Ejaculates from 36 men participating in the ICSI program were studied. All individuals had a normal physical examination, testes with normal volume, absence of varicocele, and negative semen cultures. A portion of the same processed semen sample used for ICSI was examined for each patient. The indications for ICSI included the diagnosis of male factor infertility based on clinical findings, the results of previous semen analyses showing poor sperm parameters, and/or a failure to achieve a pregnancy after ≥ 3 cycles of controlled ovarian hyperstimulation combined with intrauterine insemination therapy in cases with >5 million total motile spermatozoa (21). Couples with etiologic female factors, and women with fewer than four harvested mature oocytes were excluded from the study. Investigators assessing semen parameters and sperm DNA fragmentation were blinded to the ICSI results.

Sperm Preparation

Semen samples were collected by masturbation into sterile cups after 2 to 4 days of sexual abstinence. After liquefaction, semen characteristics and sperm parameters were assessed. Sperm concentration and progressive motility were assessed with an HTR-IVOS semen analyzer (version GS 771; Hamilton Thorne Research, Beverly, MA) and were manually monitored with fixed parameter settings (22). Motion parameters were examined after mixing the sperm suspension and loading a 5-μL aliquot into a Makler chamber (Mid-Atlantic Diagnostics Inc., Mt. Laurel, NJ). Sperm morphology was examined at $\times 1000$ magnification oil-immersion microscopy by strict criteria (23, 24) after staining with STAT III Andrology Stain (Mid-Atlantic Diagnostics, Inc.).

Motile spermatozoa were selected by the swim-up technique performed in human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA) supplemented with 0.2% human serum albumin (HSA; Irvine Scientific). The spermatozoa were washed and incubated for 60 minutes at 37°C in fresh HTF-HSA. To retrieve the highly motile fraction, the volume from the top was removed. After the separation, part of the sperm sample was used for ICSI. The remainder portion of the purified sperm population with high motility was adjusted at a concentration of $5–10 \times 10^6$ spermatozoa/mL in HTF-HSA, and was stored at –196°C without cryoprotectant until examined for DNA fragmentation and morphologic features.

Samples were thawed in a 37°C water bath for 3 minutes immediately before assessment of DNA fragmentation and sperm shape, as previously reported elsewhere (20). An aliquot of approximately 25 μL was transferred to a multiwell slide (Cel-Line/Erie, Scientific Co, Portsmouth, NH) for examination of DNA fragmentation and morphologic normality. The DNA fragmentation and morphologic characteristics were evaluated simultaneously on the same sperm cell using immunofluorescence and phase contrast, respectively. Each sample was analyzed in duplicate droplets, and the results were averaged.

TUNEL Assay

Sperm DNA fragmentation was evaluated with terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate-fluorescein nick end labeling (TUNEL) assay with the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany). The assay uses fluorescein-dUTP to label single and double DNA strand breaks, according to the manufacturer's instructions, and the assay was performed as previously published elsewhere (8). Each sperm suspension was aliquoted in 25 μL on a multiwell slide, fixed with paraformaldehyde (final concentration 2%), permeabilized with 0.1% Triton X-100, and incubated in the dark at 37°C for 1 hour in the TUNEL reaction mixture containing 0.5 IU/μL of calf thymus terminal deoxynucleotidyl transferase and fluorescein-dUTP. Negative (omitting the enzyme terminal transferase) and positive (using deoxyribonuclease I, 1 IU/mL for 20 minutes at room temperature) controls were performed in each experiment. Mounting medium for fluorescence (Vectashield; Vector Laboratories, Burlingame, CA) was added before the evaluation to protect the fluorescence. A total of 200 cells were randomly analyzed per sample in a Nikon Eclipse E600 microscope (Nikon,

Tokyo, Japan) at ×1000 magnification oil-immersion objective. Each sperm cell was classified as DNA intact (no fluorescence) or DNA fragmented (green nuclear fluorescence).

In the negative controls, none of the cells showed fluo rescent staining, but in the positive controls (treated with deoxyribonuclease I), 100% of the cells showed DNA fragmentation. The fluorescent staining of the spermatozoa persisted after several expositions under regular or fluorescence light during the counting procedure. The results were expressed as total sperm with fragmented DNA (total SFD) representing the Percentage of sperm DNA fragmentation/Total number of counted spermatozoa.

Simultaneous Examination of Normal Sperm Morphology and DNA Fragmentation

Immediately after TUNEL, and in the same droplets used for DNA fragmentation analysis, sperm morphology (without staining) was assessed as previously described elsewhere (20) in several randomly selected fields under phase-contrast microscopy (Nikon Eclipse E600) equipped with a SPOT-RT Slider digital camera (Diagnostic Instruments, Inc, Sterling, MI) using a ×1000 magnification oil-immersion objective. A total of 400 cells were evaluated in two droplets per patient. During this examination, every time a spermatozoon with normal morphology was found, the light was immediately switched to the fluorescence to determine DNA integrity. Spermatozoa were considered normal when the head had a normal shape, a symmetrical and oval head configuration, vacuoles occupying less than 20% of the head area, an acrosomal region comprising 40% to 70% of the head area, a symmetrical insertion of the tail, and absence of midpiece or neck defects (24, 25). The results were expressed as morphologically normal spermatozoa with fragmented DNA (normal SFD), representing the Percentage of sperm with normal morphologic characteristics having DNA fragmentation/Total number of morphologically normal sperm. A representative picture is shown in Figure 1.

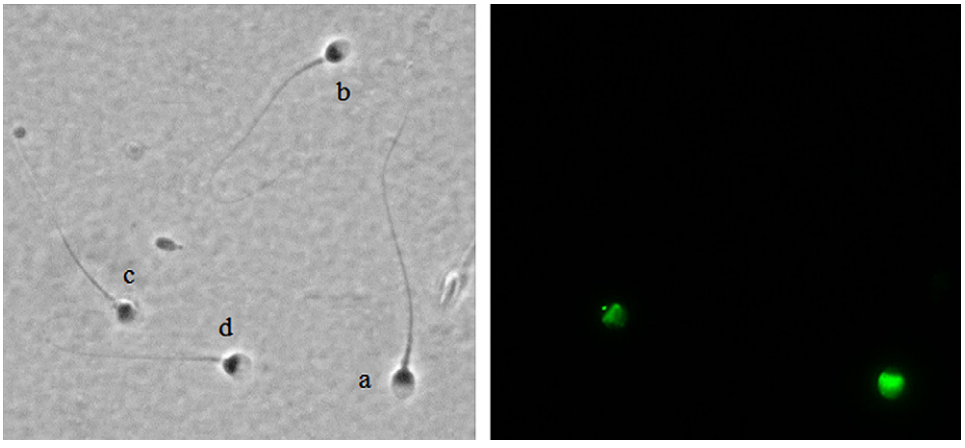
ICSI and Embryo Quality Assessment

Ovarian stimulation protocols and embryo laboratory procedures used for IVF augmented with ICSI were accomplished using previously established protocols (26). At 16 to 20 hours after microinjection, oocytes were assessed for the presence of two pronuclei (normal diploid fertilization). Seventy-two hours after oocyte retrieval, embryos were classified according to cleavage and morphology score, and then were transferred to the uterus. Morphology grading followed the criteria established by Veeck (27) with modification as follows: grade 5, no fragmentation with equal-sized cells; grade 4, <20% fragmentation with equal-sized cells; grade 3, no fragmentation with unequal-sized cells; grade 2, >20% fragmentation with unequal-sized cells; and grade 1, >50% fragmentation. An individual embryo quality score was calculated by multiplying the number of blastomeres times the morphology grade. Two to three embryos were transferred on day 3 under abdominal ultrasound guidance.

Two indexes were established to examine embryo quality (28). The first one, a mean embryo score (ES) for all embryos available for a given patient (total ES: total embryo score for the total cohort of embryos) was calculated as the Sum of scores of all available embryos/Total number of embryos of the cohort. The second one, a mean cumulative embryo score

FIGURE 1

Representative photomicrographs of the simultaneous assessment of normal sperm morphology and DNA fragmentation following swim up separation. *Left:* phase contrast. *Right:* TUNEL fluorescence. (*a*) Normal spermatozoon with DNA fragmentation; (*b*) normal spermatozoon without DNA fragmentation; (*c*) morphologically abnormal spermatozoa with DNA fragmentation; and (*d*) morphologically abnormal spermatozoa without DNA fragmentation.



Avendaño. Normal sperm, DNA status, and ICSI. Fertil Steril 2010.

per transfer (transferred ES: embryo score for the transferred embryos) was calculated as the Sum of scores of transferred individual embryos/Number of embryos transferred in the cycle.

Statistical Analysis

Data are presented as mean ± standard deviation. The studied parameters were age of female and male participants, semen parameters of original (unprocessed) samples (sperm concentration, progressive motility, and normal [stained-strict] morphology), DNA fragmentation in total spermatozoa (total SFD) and DNA fragmentation in morphologically normal spermatozoa (normal SFD) obtained from the swim-up separated motile fractions, embryo quality (mean total ES and transferred ES), and clinical pregnancy (defined as the identification of gestational sacs by vaginal ultrasound at 7 weeks).

Various independent parameters of pregnant cycles were compared with those of nonpregnant cycles using the Mann-Whitney *U* test. Spearman's Rho was used for correlation analysis. Variables that were used in the correlation analysis were female and male age, all basic semen parameters evaluated (original sperm concentration, motility, and morphology), number of oocytes inseminated, number of oocytes fertilized (diploid), mean total ES, and mean transferred ES. Two forward stepwise multiple regression analyzes were performed to assess the determinants of mean transferred ES and mean total ES including the following parameters: male and female age, number of oocytes inseminated, number of oocytes fertilized, sperm morphology (phase-contrast analysis), total SFD, and normal SFD following the swim-up technique.

In addition, receiver operating characteristic (ROC) curves were constructed to assess the ability of normal SFD and mean transferred ES to predict pregnancy. A forward stepwise logistic regression analysis was performed to assess the value of selected parameters to predict pregnancy using likelihood ratios. The evaluated parameters were male and female age, number of oocytes inseminated and fertilized, post–swim-up sperm morphology (phase-contrast analysis), total SFD and normal SFD, and mean transferred ES and mean total ES. $P<.05$ was considered statistically significant.

RESULTS

Table 1 depicts the number of samples classified in different pathologic groups according to the semen analysis results in the 36 studied patients. A high proportion of samples showed isolated teratozoospermia (moderate and severe) while a smaller percentage of individuals had low sperm concentration and/or motility, with or without concomitant abnormal sperm morphology.

We compared couples who conceived after embryo transfer (n = 15) versus those who did not achieve a pregnancy (n = 21). There were no statistically significant differences in the number of oocytes retrieved and fertilized or the mean number of transferred embryos between the two groups. Moreover, there were no statistically significant differences in female or male age, basic sperm parameters results, or the total SFD when comparing the pregnant group with the nonpregnant group. However, the normal SFD was statistically significantly higher in the nonpregnant group. These results are shown in Table 2.

TABLE 1

Classification of basic semen analysis results in the study population.

Basic semen analysis	n	%
Isolated oligozoospermia ($<20 \times 10^6$ mL)	1	2.8
Isolated asthenozoopermia (<50 % progressive motility)	1	2.8
Isolated teratozoospermia		
Moderate (5% to 9 % normal forms)	15	41.7
Severe (≤4 normal morphology)	10	27.7
Oligoteratozoospermia	3	8.3
Asthenoteratozoopermia	4	11.1
Oligoasthenoteratozoospermia	2	5.6

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It is interesting that there was no correlation between normal SFD and the percentage of normal sperm morphology in the staining or phase-contrast analysis, nor with the sperm motility and concentration.

Determinants of Embryo Quality

Nonparametric analysis demonstrated a statistically significant negative correlation between normal SFD and mean total ES ($r = –0.64$, $P<.001$) as well as between normal SFD and mean transferred ES ($r = –0.63$, $P<.001$) (Fig. 2). The multiple regression analysis ($r = 0.78$, $SE = 3.9$, $df = 29$, $P<.001$) demonstrated that the normal SFD ($B = –0.19$, $P<.001$) and female age ($B = –0.38$, $P=.03$) were the main determinants of mean transferred ES among the evaluated parameters.

On the other hand, the determinants of mean total ES were normal SFD ($B = –0.18$, $P<.001$) and the number of oocytes fertilized ($B = –0.44$, $P<.03$) by multiple regression analysis ($r = 0.73$, $SE = 3.9$, $df = 29$, $P<.001$).

Predictors of Pregnancy

The ROC curve analysis demonstrated that normal SFD and mean transferred ES are useful predictors of pregnancy probability (Fig. 3). Table 3 summarizes the ROC curve analysis results. The area under the curve (0.7 and 0.75), *P* values (<.021 and <.002), and specificity (82.6 and 91.3) for normal SFD and mean transferred ES, respectively, showed a statistically significant predictive power for pregnancy.

TABLE 2

Comparison of female and male age, original semen parameters, and total sperm with fragmented DNA (SFD) and normal SFD among pregnant and nonpregnant groups.

Group	Pregnant (n = 15)	Nonpregnant (n = 21)
Female age	34.3 ± 4.5	34.2 ± 4.4
Male age	36.5 ± 5.2	36.4 ± 4.2
Oocytes retrieved	9.9 ± 4.5	11.3 ± 5.1
Fertilization rate (%)	83 ± 16	73 ± 20
Transferred embryos	2.4 ± 0.5	2.3 ± 0.5
Semen sperm concentration ($\times 10^6$/mL)	73.6 ± 55.7	75.1 ± 65.7
Semen sperm motility (%)	49.6 ± 17.7	55.4 ± 20.9
Semen sperm morphology (%)	5.2 ± 4.8	5.5 ± 5.5
Total SFD	15.6 ± 8.5	13.3 ± 12.3
Normal SFD	18.9 ± 20.0[a]	33.8 ± 19.4[a]

[a] P=.03.

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Using ROC analysis we also determined the optimal cutoff values for pregnancy outcome prediction. According to these results, with a mean transferred ES >28, there was a 6.2 times increase in the probability of pregnancy, but with a mean transferred ES ≤28, there was a 0.5 times decrease in pregnancy. Additionally, if the normal SFD was ≤17.6%, the probability of pregnancy was 3.5 higher, but if the normal SFD was >17.6%, the pregnancy potential decreased by half.

A logistic regression analysis estimated a model to predict pregnancy (–2 log likelihood = 33.124, chi-square = 6.305 with 1 degree of freedom, P=.012). Only the mean transferred ES was included in this model (Exp B = 1.211, P=.03), probably because it was the single best independent predictor of conception. When the logistic regression analysis was performed excluding transferred ES from the list of parameters tested, the model estimated to predict pregnancy (–2 log likelihood = 34.321, chi-square = 5.109 with 1 degree of freedom, P=.024) included only normal SFD (Exp B = 0.958, P=.04) among the parameters analyzed.

DISCUSSION

The clinical introduction of ICSI has allowed many infertile men with severely affected sperm parameters the opportunity to become genetic fathers. However, ICSI is a more invasive technique than conventional IVF and bypasses the process of

FIGURE 2

Correlation between morphologically normal sperm with DNA fragmentation and embryo quality. (**A**) Scatterplot and linear regression between normal SFD (normal sperm with fragmented DNA) and mean total ES (embryo score for total embryos), r = –0.64, P<.001. (**B**) Scatterplot and linear regression between normal SFD (normal sperm with fragmented DNA) and mean transferred ES (embryo score for transferred embryos), r=–0.63, P<.001.

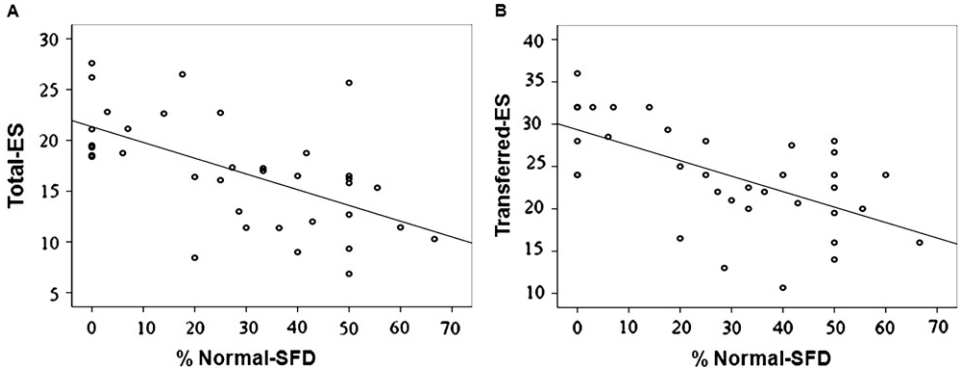

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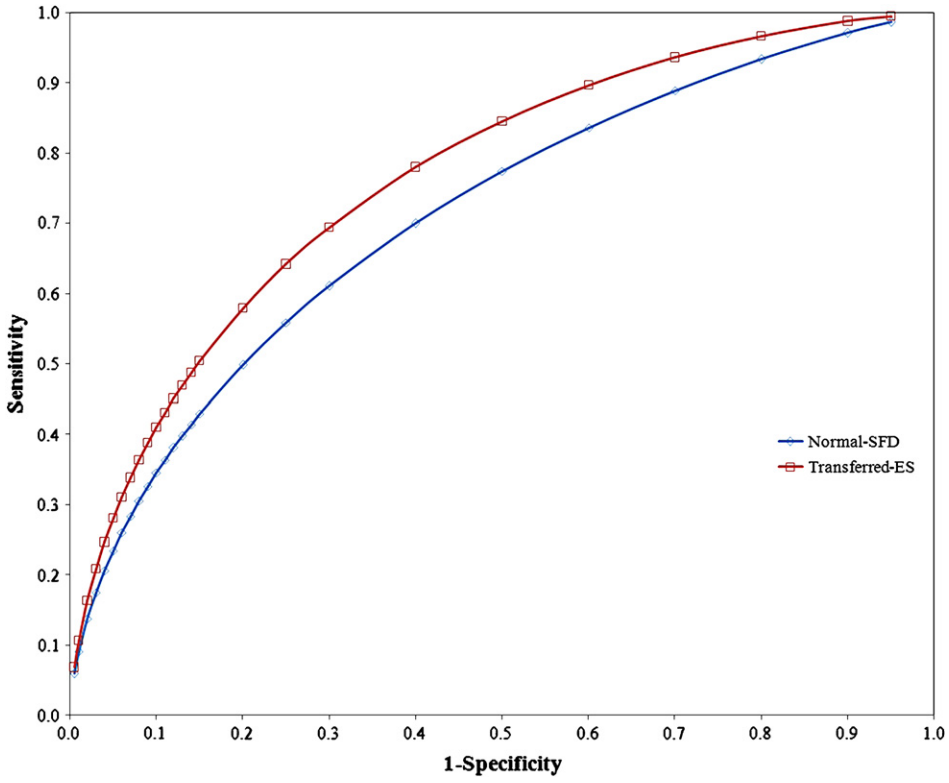
FIGURE 3

Receiver operating characteristics (ROC) curves for normal SFD (normal sperm with fragmented DNA) and mean transferred ES (embryo score for transferred embryos) to predict pregnancy. Quantitatively, the area under the curve is used to determine the accuracy of prediction. Area under the ROC curve for presence of normal SFD = 0.70 and transferred ES = 0.72.



Avendaño. Normal sperm, DNA status, and ICSI. Fertil Steril 2010.

natural sperm selection. An increased risk of chromosomal abnormalities has been shown in ICSI offspring (29, 30). In addition, a significant increase in urogenital problems in male children born after ICSI was reported in a Swedish study (31). Others have also reported an association of major cardiovascular, urogenital, chromosomal, and musculoskeletal defects with the use of ICSI (32). Because selection for ICSI is based on sperm motility and normal morphology, and because sperm with damaged DNA cannot be recognized during the routine laboratory selection procedure, the inadvertent injection of spermatozoa with DNA damage into oocytes might be determinant of some of these problems.

Although some investigators have suggested the possibility that normal sperm may show DNA fragmentation (4), we have recently demonstrated, for the first time, that infertile men can present DNA fragmentation in the morphologically normal sperm population assessed by strict criteria. In addition, a recent report of unselected couples undergoing infertility treatment showed that 15.9 % of normal sperm selected by high magnification microscopy had DNA fragmentation, thus supporting our findings (33). This prompted us to conduct the present study to correlate the presence of DNA fragmentation in morphologically normal sperm and ICSI outcome.

The first major finding of our study was the demonstration of a statistically significant negative correlation between the percentage of morphologically normal sperm with fragmented DNA (normal SFD) and embryo quality measured as the mean embryo score of total embryos (total ES) after ICSI (Fig. 2). Importantly, the data also confirmed that this association was maintained when analyzing only the transferred embryos from a given patient, as normal SFD also had a statistically significant negative correlation with the mean transferred embryo score (transferred ES). These results support the fact that the presence of normal SFD has an important negative impact on the quality of embryos after ICSI. Contrary to other studies (2, 34), we did not find a statistically significant correlation between the total SFD and embryo quality, suggesting that analyzing the morphologically normal spermatozoa subpopulation enhances the ability to predict the impact of sperm DNA fragmentation on the embryo quality. This was supported by multiple regression analysis, which revealed that normal SFD, female age, and the

TABLE 3

Summary of the receiver operating characteristic (ROC) curve analysis: power of normal sperm with fragmented DNA (SFD) and mean transferred embryo score (ES) for pregnancy prediction.

Parameter	Area under the curve	95% Confidence interval	*P* value	Cutoff point	Sensitivity	Specificity	Positive likelihood ratio	Negative likelihood ratio	Positive predictive value	Negative predictive value
Normal FD	0.70	0.53–0.84	0.02	≤17.6%	61.5	82.6	3.5	0.5	66.7	79.2
Transferred ES	0.75	0.58–0.88	0.005	>28	53.9	91.3	6.2	0.5	77.8	77.8

Avendaño. Normal sperm, DNA status, and ICSI. Fertil Steril 2010.

number of oocytes fertilized were the main determinants of embryo quality, superseding all other parameters in the prediction of mean total ES and transferred ES.

The second major finding was that the normal SFD and the mean transferred ES were statistically significant predictors of pregnancy when ROC curve analysis was performed (Fig. 3). Using optimal cutoff values and as estimated by the ROC curve analysis, both parameters showed high specificity and were accurate predictors of conception. In general, it is estimated that a test with a positive likelihood ratio (LR) >10 predicts outcome (in this case pregnancy) conclusively, whereas one with a LR 5 to 10 is a moderate and LR 2 to 5 a weak predictor of outcome (35). According to these criteria, we conclude that mean transferred ES is a moderate (LR: 6.2) and normal SFD a weaker (LR: 3.5) predictor of pregnancy in ICSI cycles. The normal SFD percentage predicts pregnancy indirectly through transferred ES, as indicated by their strong correlation and by the fact that in the logistic regression analysis only the mean transferred ES was included in the statistical model predictive of conception. In addition, when transferred ES was excluded from the list of parameters tested, the model estimated to predict pregnancy included only normal SFD. This finding supports our conclusion that normal SFD predicts pregnancy indirectly through transferred ES.

A number of studies have demonstrated an increased percentage of spermatozoa with damaged DNA in the ejaculate of infertile men as compared with healthy fertile donors (7, 16, 20). Although many studies have shown high levels of DNA fragmentation in infertile patients with abnormal sperm parameters (1, 4), it has also been reported that some patients with normal semen parameters and idiopathic infertility may present increased levels of DNA-damaged sperm (36).

In recent years, evaluation of sperm DNA fragmentation has been recognized as a predictor of the probability to conceive (4). The ability of natural conception declines as a function of the percentage of sperm with abnormal chromatin (6, 7). The negative impact of sperm DNA damage in pregnancy outcome has been shown for intrauterine insemination (IUI) (8, 9). In addition, a negative effect on fertilization (10), blastocyst development, and ongoing pregnancy (11) has been shown repeatedly in IVF.

With respect to ICSI, several studies have reported discrepancies on the impact of sperm DNA fragmentation. Some groups have showed a statistically significant negative association between the percentage of sperm with fragmented DNA and the ICSI fertilization rate (15), but others have reported no difference in the fertilization rate but a negative effect in the pregnancy rate (16) or embryo quality (17). Also, increased values of sperm DNA fragmentation have been proposed as a possible cause of low quality of postimplantation embryo and spontaneous abortion (2, 12, 34). On the other hand, some studies have failed to show any effect of sperm DNA fragmentation on ICSI outcomes (9, 14). These studies have evaluated DNA fragmentation in the total sperm

population (morphologically abnormal and normal spermatozoa), but during the ICSI procedure only the good-shaped sperm are selected for injection. This is why a DNA fragmentation analysis of the normal sperm subpopulation is a better predictor of embryo quality and pregnancy outcomes than an analysis the entire swim-up sample.

Notwithstanding these observations, spermatozoa with damaged DNA may retain the potential to fertilize and produce a viable embryo (2, 37). Although the oocyte has the capability to repair damaged paternal DNA, this ability is limited and depends on the grade of damaged DNA (38). Such damage can lead to a predisposition to mutations in the developing embryo with the potential to induce disease in the offspring (39).

The putative effect of sperm DNA damage on the health of future generations is not yet known. Some studies using animal models have indicated that sperm damaged by chemotherapeutic agents transmit heritable translocations, mutations and malformations to the next generation (40). The use of spermatozoa with damaged DNA through ICSI have indicated that genetic and epigenetic changes during pre-implantation may occur, leading to altered fetal development and, as consequence, offspring with aberrant growth, behavior, early aging and tumors (19). Functional bovine sperm with damaged DNA can normally fertilize the oocyte and no significant effect is observed during the first cleavages of the fertilized oocyte. However, a significantly negative effect in further embryo development can be observed as evidenced by embryo fragmentation, apoptosis and aberrant or no signs of mitotic spindle formation (38).

Previous studies have revealed a threshold of 15% to 20% of sperm with fragmented DNA (evaluated by TUNEL) over which pregnancy outcome is lower when ICSI is used (16, 34). Our results, however, showed that the percentage of total sperm with fragmented DNA (total SFD) is not associated with ICSI outcome. Instead, the proportion of morphologically normal sperm with fragmented DNA (normal SFD) was a good predictor of embryo quality and a weak predictor of pregnancy. Our results suggest that swim-up samples containing more than 17.6 % of morphologically normal sperm with fragmented DNA are associated with a higher possibility of generating poor quality embryos and are less likely to result in pregnancy.

In conclusion, although it is not possible to assess DNA integrity in the spermatozoa to be injected during ICSI, the evaluation of DNA fragmentation in morphologically normal sperm obtained from the motile fraction is apparently, albeit indirect, the closest way to reflect it. We have demonstrated that the DNA fragmentation in morphologically normal spermatozoa has a statistically significantly negative effect on embryo quality and pregnancy outcome in ICSI patients.

Even considering the relatively low number of samples analyzed in this study, we have demonstrated that the evaluation of sperm DNA fragmentation when assessing the morphologically normal cell population is useful in predicting embryo quality and it is also a predictor of pregnancy. We propose that this new way of evaluating spermatozoa could be used as a more reliable methodology in predicting ICSI outcomes.

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DNA Fragmentation in Morphologically Normal Spermatozoa: How Much Should We Be Concerned in the ICSI Era?

Review

CONRADO AVENDAÑO* AND SERGIO OEHNINGER†

*From *Nascentis Medicina Reproductiva, Córdoba, Argentina; and the †Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, Norfolk, Virginia.*

ABSTRACT: Intracytoplasmic sperm injection (ICSI) has revolutionized the treatment of male infertility. However, there are still unanswered questions about the safety of this technique. During ICSI, only morphologically normal and motile spermatozoa are typically used to fertilize an oocyte. We recently reported that in infertile men, spermatozoa with apparently normal morphology may have DNA fragmentation. This finding consequently raised the possibility that spermatozoa with normal-shaped appearance but with DNA fragmentation could be mistakenly selected to fertilize oocytes during ICSI. This concern became more clinically significant following the subsequent finding that the presence of an increased proportion of normal spermatozoa with damaged DNA was negatively associated with embryo quality and pregnancy outcome after ICSI. Herein, we propose and discuss the hypothesis that the examination of DNA integrity in the subpopulation of highly motile (hence viable) and morphologically normal cells (and not in the total sperm population) may provide optimized information in prediction of ICSI success. More importantly, this new way of evaluation may provide reassurance about genomic normalcy and minimal risk of transmission of genetic disease and guide the development of improved methods of selection of spermatozoa with intact DNA to be used in assisted reproduction.

Key words: Morphology, pregnancy.
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Sperm DNA integrity is crucial for the maintenance of paternal reproductive potential. It has been shown that spermatozoa which carry an altered genome are able to achieve fertilization, thereby posing concerns about the transmission of abnormal genetic material to the offspring, either during natural conception or following the use of assisted reproductive technology (ART) (Van der Zwalmen et al, 1991; Janny and Menezo, 1994; Ahmadi and Ng, 1999; Tesarik et al, 2004; Barroso et al, 2009).

Intracytoplasmic sperm injection (ICSI) bypasses the physiologic selection barriers at fertilization (Oehninger and Gosden, 2002). In ICSI, a single spermatozoon is selected and injected into a mature oocyte's cytoplasm (Palermo et al, 1992). Sperm selection for ICSI is typically based on motility and morphology attributes, without information about the chromosomal/DNA status. Indeed, concerns have been raised regarding possible use of spermatozoa with chromosomal anomalies and/or DNA damage during ICSI (Seli and Sakkas, 2005; Aitken and De Iuliis, 2007). Moreover, although there are positive correlations between the degrees and types of teratozoospermia and sperm chromosomal anomalies/DNA damage (Moskovtsev et al, 2009; Tang et al, 2010), sperm morphology per se is not a strong predictor of aneuploidies or DNA fragmentation (Celik-Ozenci et al, 2004).

Many studies have shown that infertile men have higher levels of DNA strand breaks and other types of DNA damage than fertile donors (Zini et al, 2001; Benchaib et al, 2003; Sergerie et al, 2005). Infertile patients with varying degrees of oligoasthenoteratozoospermia frequently show increased levels of DNA fragmentation, and it has also been reported that even infertile patients with normozoospermic parameters can have higher values of DNA damage than fertile controls (Saleh et al, 2002).

Couples with poor semen quality and/or those who have failed conventional in vitro fertilization (IVF) are nowadays best treated by ICSI (Oehninger, 2001). Consequently, these types of patients are confronted with a higher risk of selection of DNA-damaged spermatozoa for ICSI. It has been proposed that the use of ''invisible damaged'' spermatozoa could result in fertilization failure, impaired normal embryo develop-

Correspondence to: Dr Sergio Oehninger, Jones Institute for Reproductive Medicine, 601 Colley Ave, Norfolk, VA 23507 (e-mail: oehninsc@evms.edu).

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ment, reduced implantation or pregnancy rate, and/or even transference of damaged DNA to the new generation (Aitken and De Iuliis, 2007).

The use of ICSI has become the most common oocyte fertilization method (compared with standard IVF insemination), being performed in 64% of IVF cases in the United States (Centers for Disease Control and Prevention, 2009) and with an increased worldwide application including Europe, which reported 63% ICSI usage (Nyboe Andersen et al, 2009). In addition, recent reports have suggested that some birth defects occur more often among infants conceived with ART (Reefhuis et al, 2009). Because spermatozoa with normal morphology may have DNA damage (Avendaño et al, 2009), it can be speculated that damaged sperm may be ''inadvertently'' used in ICSI, resulting in negative untoward embryonic/fetal effects.

Hence, the objectives of this article are to 1) review the use of DNA fragmentation tests for clinical decision making in ART and 2) promote further discussion on the selection of morphologically normal sperm with intact DNA for ICSI. The main conclusions are that there is a risk of microinjecting DNA-damaged sperm at the time of ICSI, with unknown (at this time) untoward embryonic effects and that the development of newer methods to select DNA-intact sperm should be sought vigorously.

Normal Sperm Morphology and DNA Fragmentation

Sperm morphology has been recognized as an excellent predictor of the outcome of natural conception, intrauterine insemination, and conventional IVF therapies (Oehninger and Kruger, 1995). However, the percentage of normal sperm morphology in the unprocessed ejaculate or even after separation of the fraction with highest motility has no impact on the outcome of ICSI (Nagy et al, 1995; Oehninger et al, 1995). Recently, however, the morphologic normalcy of spermatozoa to be used in ICSI has been established as an important factor for achieving pregnancy, as demonstrated by the use of high-magnification microscopic methods of sperm selection for microinjection (Berkovitz et al, 2005). This point is extremely important because in this procedure, typically only morphologically normal sperm are selected, regardless of the absolute amount present in the sample (French et al, 2010).

The impact of sperm DNA integrity on ICSI outcome has been studied (in the total sperm population) using a variety of techniques, with contradictory results. Lopes et al (1998) showed a significant and negative association between the percentage of sperm with fragmented DNA and fertilization rate after microinjection. However, others reported no difference in fertilization rate but a negative effect on the pregnancy rate (Benchaib et al, 2003). Also, increased levels of sperm DNA fragmentation have been proposed as a possible cause of low-quality embryos and spontaneous abortion (Evenson et al, 1999; Tesarik et al, 2004; Borini et al, 2006). On the other hand, other researchers could not find any effect of high levels of sperm DNA fragmentation on ICSI outcomes (Gandini et al, 2004; Bungum et al, 2007; Nicopoullos et al, 2008).

Collins et al (2008) investigated a possible association between DNA damage and ART outcome. The researchers identified 13 studies involving 2161 IVF/ICSI treatments that provided DNA test data. A small but significantly increased risk of failed pregnancy (diagnostic odds ratio [OR], 1.44; 95% confidence intervals [CI], 1.03–2.03) was found, but the researchers concluded that such tests were not clinically useful in discriminating couples who would conceive with treatment; furthermore, a subgroup analysis considering the type of DNA test, patient characteristics, and treatment modality did not impact this conclusion. These findings may be explained by the fact that all cited studies evaluated DNA fragmentation in the total sperm population (morphologically normal and abnormal spermatozoa).

Recently we reported that highly motile and morphologically normal sperm (examined by strict criteria) may have DNA fragmentation (Avendaño et al, 2009). This finding was extremely significant in infertile men with severe teratozoospermia (all studied patients had some degree of DNA fragmentation, ranging from 20% to 60% affected cells) and even present, albeit in much lower proportions, in subfertile patients with borderline to normal semen parameters (25% of those men had some degree of DNA damage in morphologically normal spermatozoa). On the other hand, none of the fertile men (donors) examined as controls had any morphologically normal spermatozoa with DNA damage.

Even more clinically significant was the subsequent finding that the presence of an increased proportion of normal spermatozoa with damaged DNA was negatively associated with embryo quality and pregnancy outcome after ICSI (Avendaño et al, 2010). Because typically only motile and morphologically normal sperm are selected for ICSI, the selection and injection of morphologically abnormal spermatozoa are less likely to occur. In consequence, we postulate that the evaluation of DNA fragmentation in the total sperm population (normal and abnormal) is not the best way to assess the possibility of success with ICSI. Instead, the evaluation of DNA integrity in morphologically normal spermatozoa after sperm selection is a better approach to examine sperm DNA fragmentation and any potential impact on the ICSI procedure. A representative picture of possible clinical scenarios is shown in the Figure.

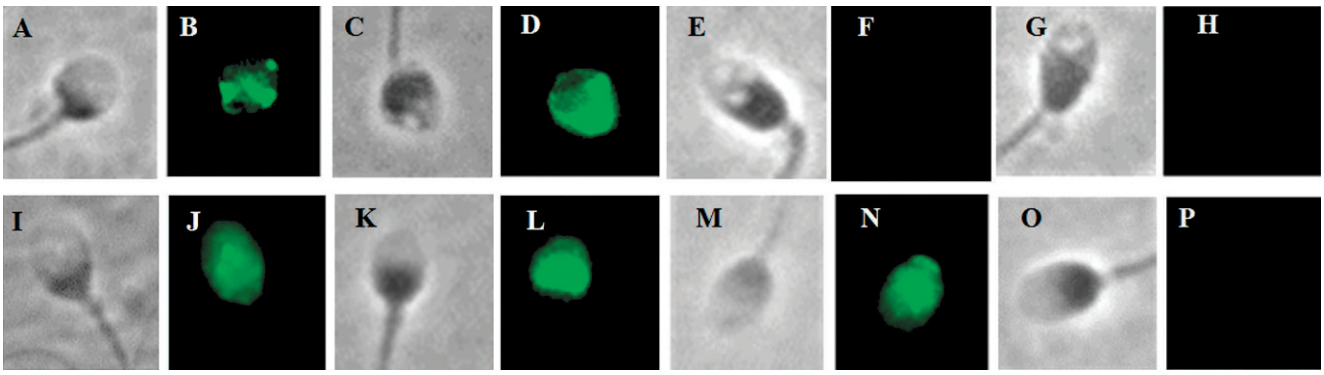

Figure. Representative photomicrographs of the simultaneous assessment of sperm morphology (phase contrast: **A, C, E, G, I, K, M, O**) and DNA fragmentation (fluorescence by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling: **B, D, F, H, J, L, N, P**) in the separated motile fractions. Upper lane: Abnormal sperm (phase contrast) that would not be typically selected for intracytoplasmic sperm injection (ICSI) owing to poor morphology, with fragmented DNA (with fluorescence; **A** and **B, C** and **D, E** and **F**) or without DNA fragmentation (no fluorescence; **G, H**). Lower lane: Morphologically normal spermatozoa that would be typically selected for ICSI, with DNA fragmentation (with fluorescence; **I** and **J, K** and **L, M** and **N**) or without DNA fragmentation (no fluorescence; **O, P**).

Sperm Parameters, Sperm DNA Fragmentation, and ICSI Outcome

We evaluated 49 semen samples from infertile men undergoing ICSI, and an aliquot of the same processed sample used for microinjection was simultaneously tested for morphology and terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL). The Table presents a correlation analysis of the percentage of total sperm with fragmented DNA (morphologically normal and abnormal sperm) and the percentage of morphologically normal sperm with fragmented DNA, with the sperm parameters after swim-up separation and ICSI outcomes. A significant and negative correlation ($P = .004$) was found between total sperm with fragmented DNA and sperm motility after swim-up separation. On the other hand, no correlation was observed between the proportion of morphologically normal sperm with fragmented DNA and any of the seminal parameters examined. Likewise, there was no correlation between the percentage of sperm with DNA fragmentation in the morphologically normal population and the percentage of total sperm with fragmented DNA. This finding reinforces the observation that the presence of morphologically normal sperm with fragmented DNA is independent of the other semen parameters.

There was no direct correlation between the percentage of total sperm with fragmented DNA and ICSI outcomes. In contrast, when morphologically normal spermatozoa with fragmented DNA were evaluated, significant and negative correlations were found with early embryo cleavage rate ($r = -.297$; $P = .038$), mean embryo quality at culture day 2 ($r = -.353$; $P = .013$), and mean embryo quality at culture day 3 ($r = -.490$; $P < .001$). In our recent communication (Avendaño et al,

Table. *Correlations between the percentages of total sperm with fragmented DNA and morphologically normal sperm with fragmented DNA for basic sperm parameters and ICSI outcomes*[a]

	Total Sperm With Fragmented DNA		Normal Sperm With Fragmented DNA	
Parameter	*r*	*P*	*r*	*P*
Sperm concentration	−.121	.409	.247	.086
Sperm motility	−.401	.004[b]	.125	.391
Sperm morphology	−.177	.225	.154	.289
Fertilization rate	−.020	.890	−.226	.065
Early embryo cleavage	.044	.763	−.297	.038[b]
Mean embryo quality day 2	−.016	.914	−.353	.013[b]
Mean embryo quality day 3	.004	.979	−.490	<.001[b]
Total sperm with fragmented DNA	...	...	.048	.745
Normal sperm with fragmented DNA	.045	.745	...	...

[a] n = 49 patients/couples. Motile spermatozoa selected by swim-up (from the same sample used for intracytoplasmic sperm injection [ICSI]) were used to perform a simultaneous assessment of normal sperm morphology (using phase contrast) and DNA fragmentation (switching to fluorescence and using terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling) using the ×1000 oil immersion objective. Spearman's ρ test was used.

[b] Statistically significant.

2010), we also observed that when the percentage of normal sperm with fragmented DNA was 17.6% or lower, the likelihood of pregnancy was 3.5 times higher than when above this level. We concluded that the DNA fragmentation of morphologically normal sperm not only negatively impacted embryo quality but also the probability of pregnancy in ICSI cycles.

These results strengthen the conclusion that the assessment of DNA fragmentation in morphologically normal sperm appears to be a more accurate way to evaluate the paternal genome integrity impact in ICSI outcomes and that the evaluation of DNA fragmentation in the total sperm population is not as predictive.

State of the Art and Immediate Challenges

The most common types of identified sperm DNA damage are 1) single or double DNA strand breaks, 2) loss of a base to create a basic site, 3) chemical modification of a base by, for example, oxidation or alkylation, 4) interstrand or intrastrand cross-linkage, and 5) DNA–protein cross-links (Aitken and De Iuliis, 2007; Aitken et al, 2009). Moreover, it has been proposed that the possible origins of this DNA damage may be multiple and could be the result of 1) presence of postmeiotically initiated abortive apoptosis, 2) unresolved strand breaks occurring during spermiogenesis to relieve the torsional stresses associated with chromatin remodeling, and 3) direct forms of oxidative stress caused by endogenous or exogenous agents (Aitken and De Iuliis, 2010).

It has been shown that defective spermatozoa from infertile men display markers of cellular ''dysmaturity,'' including the retention of excess residual cytoplasm, persistent nuclear histones, poor capacity to bind to the zona pellucida, and disrupted chaperone content (Huszar et al, 1994; Oehninger, 2003; Aitken and De Iuliis, 2010). Diminished sperm maturity is frequently seen in oligoasthenoteratozoospermia samples and has been associated with the possible presence of apoptosis and associated DNA fragmentation (Oehninger et al, 2003; Cayli et al, 2004). Therefore, sperm DNA fragmentation may be the consequence of multifactorial causes; however, notwithstanding its origin(s), the true impact on reproduction needs to be further defined.

Based on the current knowledge status, immediate research needs to be directed to answer a few critical questions.

Which is the Best Clinical Diagnostic Test to Assess DNA Damage?

Most of the techniques used to detect DNA damage in spermatozoa provide very little information about the nature of the lesions (Aitken et al, 2009). In addition, there is no consensus about which test offers more diagnostic benefit that could result in optimized clinical management. A variety of tests have been introduced, including TUNEL (Gorczyka et al, 1993), comet (Hughes et al, 1996), in situ nick translation (Tomlinson et al, 2001), DNA breakage detection fluorescence in situ hybridization (Fernández et al, 2000), sperm chromatin dispersion test (Fernández et al, 2003), and sperm chromatin structure assay (Evenson et al, 1980).

Some of these tests measure DNA damage directly, such as TUNEL or comet at neutral pH; others measure DNA damage after denaturation steps, such as sperm chromatin structure assay, sperm chromatin dispersion test, and comet at acid or alkaline pH (Sakkas and Alvarez, 2010). Indirect methods measure DNA susceptibility to denaturation after exposure to acid conditions (Evenson and Wixon, 2005). However, it has been recently suggested that these methods evaluate acid-labile sites and should not have a significant impact on the formation of male pronucleus because the intracellular pH of the oocyte is approximately 7.0 (Sakkas and Alvarez, 2010).

The TUNEL assay measures actual single- and double-strand DNA damage in human sperm, without the use of previous DNA denaturation steps, and is probably recommended as a test that measures ''real'' DNA status (as compared with ''susceptibility'') (Alvarez, 2005; Aitken et al, 2009). Nevertheless, it has been reported that results of different sperm DNA damage tests are correlated to some degree, pointing to a common origin of the damage (Aitken et al, 2009). In addition, and importantly, these tests do not diagnose absolute numbers of DNA breaks and/or are not able to quantify the amount or type of damage in sperm. Therefore, urgent research is needed to determine the best test used for screening for the presence of ''clinically relevant'' DNA damage.

What Are the True Functional Consequences of Sperm DNA Damage?

Although irrefutable human data are lacking, sperm DNA damage has been linked to poor rates of conception, impaired embryonic development, increased incidence of miscarriage, and appearance of various kinds of morbidity in the offspring, including childhood cancer (Aitken and De Iuliis, 2010). Studies in animal models are helpful to understand the real biologic impact of sperm DNA fragmentation. It has been revealed that sperm with damaged DNA can successfully fertilize the oocyte (Twigg et al, 1998). However, the use of DNA-damaged sperm reduced the rates of preimplantation and embryo development and reduced the number of offspring (Hourcade et al, 2010).

Different origins of DNA fragmentation may produce dissimilar effects. For example, although scrotal heat stress reduced the pregnancy and implantation rates, radiation exposure significantly increased the embryonic resorption rate compared with a control group (Hourcade et al, 2010). In addition, long-term multigenerational consequences, such as aberrant growth, premature aging, abnormal behavior, and mesenchymal tumors, have been documented (Fernandez-Gonzalez et al, 2008). It is expected that other novel assays may lead us to better understand the nature of human sperm DNA lesions and quantity and elucidate their genetic repercussions and their postembryonic effects.

What Is the Human Oocyte Capacity for DNA Repair?

The number of de novo structural chromosome aberrations of male descent are increased among children born after ICSI (Bonduelle et al, 2002). Although the exact etiology of structural chromosomal aberrations is unknown, misrepair of double-strand DNA breaks appears to be a prerequisite. Structural chromosomal aberrations such as dicentric chromosomes, reciprocal translocations, and acentric fragments represent failure of oocyte repair mechanisms (Richardson and Jasin, 2000). Homologous recombination and nonhomologous end joining are well-characterized mechanisms of the DNA repair machinery (Wyman and Kanaar, 2006). In the mouse, mutagenic exposure (radiation-induced sperm DNA lesions) during late spermatogenesis was shown to induce damage that persisted for at least 7 days in the fertilizing sperm and the maternal genotype played a major role in determining the risks for pregnancy loss and frequency of offspring with chromosomal defects of paternal origin depending on the efficiency of oocyte DNA repair capacity (Marchetti et al, 2007).

One surveillance mechanism that protects cells from double-strand breaks uses enzymes that recognize and phosphorylate proteins surrounding the break, principally histone γH2AX. Using a human-murine heterologous ICSI model and gamma H2AX immunostaining protein, it has been reported that it is possible to estimate the absolute amount of double-strand breaks after sperm selection for ICSI and sperm chromatin remodeling in the oocyte. This method may provide a highly sensitive single-sperm analysis to study questions on sperm DNA integrity and the oocyte response in humans (Derijck et al, 2007).

Undoubtedly, further research is needed to determine the competence of the human oocyte to repair DNA damage carried by the fertilizing spermatozoon. To better answer this question, the types and intensity of DNA damage per sperm cell need to be further characterized. Moreover, it needs to be determined whether the oocyte competence for repair under natural conditions is similar to that seen in oocytes following gonadotropin stimulation for IVF. The possibility exists that "dysfunctional" oocytes recovered in IVF might have compromised competence for DNA repair, thereby increasing risks for untoward effects.

Can We Improve the Selection of DNA-Intact Sperm for ICSI?

During ICSI, the natural process of sperm selection is superseded by the embryologist and is based on sperm morphology characteristics within the limits of microscopic magnification, as well as on the availability of motile spermatozoa. Recent studies showed that spermatozoa selected with normal overall morphology and nuclear features using specialized high-magnification microscopy may lead to higher fertilization and implantation and number of live births (Berkovitz et al, 2005). Despite these improvements, concerns remain regarding insemination of spermatozoa with chromosomal aneuploidies and/or DNA fragmentation during ICSI.

Several procedures can be used for selection of mature spermatozoa for ICSI. These procedures include 1) sperm density gradients based on sperm mass to volume (Morrell et al, 2004), 2) swim-up based on sperm motility (Lopata et al, 1976), 3) glass wool filtration based on self-propelled movement of the spermatozoa and the filtration effect of glass wool (Henkel and Schill, 2003), 4) hyaluronic acid–binding method based on the presence of a hyaluronic acid receptor (Jakab et al, 2005), 5) sperm magnetic sorting with annexin V microbeads based on apoptotic markers such as the presence of externalized phosphatidylserine to the surface membrane of spermatozoa (Grunewald et al, 2001) (the safety of this method has not been fully elucidated [Grunewald et al, 2007]), and 6) electrophoretic isolation of spermatozoa based on their size and charge (Ainsworth et al, 2005).

There is compelling clinical evidence that supports the efficiency of these procedures for selection of sperm with superior morphology and motility, and to some degree, reduction of "dysmature" and probably "apoptotic" cells. Unfortunately, none of these techniques provides the complete removal of the cells with damaged DNA. A major issue is that sperm DNA fragmentation evaluation in live cells is not possible with the techniques currently available.

Recent reports have addressed this issue with novel techniques. Confocal light absorption and scattering spectroscopic microscopy is an optical imaging technique capable of noninvasively determining the integrity of subcellular organelles. It has been proposed to be able

to identify cells with damaged DNA (Itzkan et al, 2007). However, this new technology has not been used in sperm cells yet. Huser et al (2009) reported that Raman spectroscopy of DNA packaging in individual human sperm cells distinguishes normal from abnormal cells. The researchers examined the spectra obtained from single sperm cells of a healthy male by micro-Raman spectroscopy in an effort to determine if there are DNA- or protein-related differences in the Raman spectra of sperm chromatin that correlate the shape of the nucleus (normal vs abnormal) with protein content and DNA conformation. Although the study was conducted on fixed amembraneous cells to minimize sample handling issues during optimization of the technical procedures, the researchers claimed that this analysis can be extended to living sperm cells, for example, by the combination of laser tweezers and Raman spectroscopy or the rapid analysis of cells by coherent Raman techniques.

Gianaroli et al (2010) used polarized light that permitted microscopic analysis of the pattern of birefringence in the human sperm head to examine the impact of acrosomal status on ICSI outcome. The researchers concluded that spermatozoa which have undergone the acrosome reaction seem to be more prone to supporting the development of viable ICSI embryos. It remains to be determined if there is a relationship between this sperm functional capacity (acrosome reaction) and DNA normalcy. In this regard, Lee et al (2010), using magnetic-activated cell sorting for sperm preparation, showed reduction of spermatozoa with apoptotic markers and improvement in the acrosome reaction in couples with unexplained infertility.

For these reasons, the search for novel technology methods that select morphologically normal and DNA-intact sperm must continue. Concerns regarding the safe use of ART for the treatment of infertility have been voiced for several years; these concerns include epigenetic-related conditions. However, to date, the vast majority of children conceived using these techniques are apparently normal (Grace and Sinclair, 2009). The performance of well-designed longitudinal studies on specific cohorts of ART-conceived children is therefore mandatory.

Conclusions

Male fertility potential is extremely difficult to predict on the basis of a single sperm variable. Clearly, there are many male and female factors contributing to the successful establishment of a viable pregnancy. Within the ART setting, the type and degree of DNA damage experienced by the spermatozoa (presence of adducts, degree of single- and double-stranded DNA fragmentation, association with genetic and/or epigenetic defects), whether the result of direct oxidative damage, apoptosis, or another cause, can have a profound impact on clinical outcomes. Therefore, the use of sperm cells with ''invisible'' damage should be prevented in the ART setting. To date, it is not possible to assess DNA integrity in the spermatozoa to be injected during ICSI, and current sperm separation techniques are efficient in a limited fashion. We propose that the evaluation of DNA integrity in morphologically normal spermatozoa after sperm selection is a better approach to evaluate the impact of sperm DNA fragmentation on ICSI outcome than the assessment of the total sperm population. It will be important to establish the exact nature of the DNA lesions as well as their intensities. This new way of evaluation may guide the development of improved methods of selection of spermatozoa with intact DNA.

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ANEXO III

Comunicaciones a Congresos

- **Avendaño C**. "Estudio de fragmentación de ADN en espermatozoides con diferentes morfologías. Valor predictivo en tratamientos de ICSI". VI Congreso Argentino de Andrología Sociedad Argentina de Andrología (SAA) y V Congreso Asociación Iberoamericana de Sociedades Andrológicas (ANDRO), Buenos Aires, Argentina, 2012.

- **Avendaño C**, Mata A, Juárez Villanueva A, Battello N, Sánchez Sarmiento C, Oehninger S. "DNA fragmentation of sperm subpopulations with different head morphology. Implications for ICSI treatment". American Society for Reproductive Medicine 67th Annual Meeting, Orlando, Florida, USA, 2011. Resumen publicado en Fertility & Sterility, Sep 2011, Vol. 96(3), Supplement,: S160.

- **Avendaño C**, Bocca S, Oehninger S. "The incidence of DNA fragmentation in morphologically normal sperm is a better predictor of ICSI outcome than the evaluation of DNA fragmentation in the total sperm population". American Society for Reproductive Medicine 67th Annual Meeting, Orlando, Florida, USA, 2011. Resumen publicado en Fertility and Sterility Vol. 96 (3), Supplement: S158

- **Avendaño Conrado**, Franchi Anahí, Oehninger Sergio "La Evaluación Simultanea De Fragmentación De ADN En Espermatozoides De Forma Normal Permite Predecir La Calidad Embrionaria En Pacientes De ICSI". Sociedad Argentina de Medicina Reproductiva. XIII Congreso. Buenos Aires, Argentina, 2009. Resumen publicado en Reproducción, 2009. Marzo, vol 24, suplemento 1: 18.

- **Conrado Avendaño**, Anahí Franchi, Sergio Oehninger "DNA fragmentation in morphologically normal sperm is associated with decreased embryo quality and pregnancy outcome in ICSI treatment". American Society for Reproductive Medicine 64th Annual Meeting, San Francisco, USA, 2008. Resumen publicado en Fertility & Sterility, Sep 2008 Vol. 90, Supplement: S195.

- **Avendaño C**, Franchi A, Taylor S, Morshedi M, Bocca S, Oehninger S "DNA fragmentation in morphologically normal human spermatozoa from teratozoospermic patients". 24th Annual Meeting of European Society of Human Reproductive and Embryology, Barcelona 2008. Resumen publicado en Human Reproduction, 2008 July, vol 23, supplement 1: i76.