Beyond IUI, IVF and ICSI - New developments in the selection and use of sperm for ART
Special Interest Group Andrology

1 July 2012
Istanbul, Turkey
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Organised by
the Special Interest Group Andrology
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Course coordinators

Herman Tournaye (Belgium) and Roelof Menkveld (South Africa)

Course description

This advanced course focuses on novel methods for sperm selection for ART and on troubleshooting common and less common sperm problems in the ART lab.

Target audience

All clinical, scientists and laboratory workers, working with of having an interest in sperm selection or preparation methods for ART procedures (IVF/ICSI).
Scientific programme

Sperm selection
Chairs: Roelof Menkveld (South Africa) & Sheena Lewis (United Kingdom)

In this session the focus is on comparing novel methods for sperm selection with well-established low-tech methods

09.00 - 09.30 Go with the flow. Micro-fluidics and beyond – Gary Smith (USA)
09.30 - 09.45 Discussion
09.45 - 10.15 Selecting spermatozoa; potential roles for electrophoresis and pharmacological enhancement – John Aitken (Australia)
10.15 - 10.30 Discussion
10.30 - 11.00 Coffee break
11.00 - 11.30 Magnificent? High-power optical selection methods (IMSI vs ICSI) - Laura Rienzi (Italy)
11.30 - 11.45 Discussion
11.45 - 12.15 Separating the wheat from the chaff. Selection on the basis of sperm surface markers – Liliana Ramos (The Netherlands)
12.15 - 12.30 Discussion
12.30 - 13.30 Lunch

Sperm@work
Chairs: Herman Tournaye (Belgium) & Jose Castilla (Spain)

In this session the focus lies on troubleshooting common and less common sperm problems in the ART lab

13.30 - 14.00 May the force be with you. Using immotile sperm in ART – Greta Verheyen (Belgium)
14.00 - 14.15 Discussion
14.15 - 14.45 No sperm today. Unexpected azoospermia at OPU - Raphael Ron-El (Israel)
14.45 - 15.00 Discussion
15.00 - 15.30 Coffee break
15.30 - 16.00 ‘The beauty and the beast’. When the sperm fails to activate the oocyte: what’s next? – Raaga Mansour (Egypt)
16.00 - 16.15 Discussion
16.15 - 16.45 Longing for a girl: Gender selection by natural methods – Annet Noorlander (The Netherlands)
16.45 - 17.00 Discussion
17.00 - 18:30 SIG-Andrology business meeting
Go With The Flow. Microfluidics And Beyond

Gary D. Smith, Ph.D.
Professor
Director of Reproductive Sciences Program
Director of Consortium for Stem Cell Therapies
Departs of OB/GYN, Physiology, and Urology
smithgd@umich.edu

Disclosures

Work within our laboratories on microfluidics for andrology have been supported by the NIH, USDA, State of Michigan, and Coulter Foundation.

Patents for microfluidic technologies for ART have been issued.

I was a major stockholder of Incept Biosystems, a start-up company working in the area of microfluidics and ART. Incept was purchased by Origio.

I am on the Scientific Advisory Board of Origio.

Learning Objectives / Outline

1. Introduction to microfluidics
2. Microfluidics for isolation of motile sperm
3. Microfluidics for micro-insemination
4. Microfluidic integrations and beyond
5. Concluding remarks
In Vitro Fertilization: A Micro-Process

In Vitro Fertilization of the Past and Present

In Vitro Fertilization and Embryo Culture:
- Media have changed substantially
- Processes have changed minimally (ICSI / extended culture)
- Hardware / related environments remain the same

New Tools for Cell Based Treatment, Diagnosis, and Biology
Microfluidics

- study of physical principles of fluid behavior in a microenvironment and its application to chemistry, molecular biology, and cell biology

1) Size / Mechanical Advantages
2) Microenvironment / Physiological Advantages

Turbulent Versus Laminar Flow

Fluid at the microscale exhibits laminar flow
Laminar flow is streamline and predictable
IVF: A Multi-Step Process With Stepwise Inefficiencies

Optimization of each step → Greater overall success

Microfluidics ideal for Single Cells, Small Volumes & MicroPhysiology

Could Microfluidics Be Useful In Isolation of Motile Sperm?

Theory: In a microfluidic device, motile sperm would be able to deviate from their initial stream-of-flow, cross the inter-streamline, and be isolated and enriched.

Initial stream-of-flow

Inter-streamline

Microscale Integrated Sperm Sorter

“Vision”

What was needed?
- Sperm injection port
- Fluid reservoir
- Sperm collection port
- Pump and/or power source
Polydimethylsiloxan and Sperm Survival

![Graph showing sperm motility (%)](image)

Initial Untreated PDMS Latex Overnight

- a, b, c: P<0.05

Microfluidic Sperm Separation

![Image of microfluidic device](image)

Live Sperm Go Right Dead Sperm Go Straight

Microfluidic Motile Sperm Isolation

![Image of microfluidic device](image)
Microfluidic Human Sperm Sorting

Schuster et al., Reprod Biomed Online; 2003

Initial Sorted

0 20 40 60 80 100
Sperm Motility (%)

n=10

a,b; P<0.05

Isolation of Motile Spermatozoa from Debris Laden Samples

Schuster et al., Reprod Biomed Online; 2003

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inlet</th>
<th>Outlet</th>
<th>Outlet</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1:10</td>
<td>64:1</td>
<td>96</td>
</tr>
<tr>
<td>B</td>
<td>1:10</td>
<td>100:1</td>
<td>99</td>
</tr>
<tr>
<td>C</td>
<td>1:10</td>
<td>32:1</td>
<td>95</td>
</tr>
<tr>
<td>D</td>
<td>1:10</td>
<td>12:1</td>
<td>97</td>
</tr>
<tr>
<td>E</td>
<td>1:10</td>
<td>34:1</td>
<td>99</td>
</tr>
<tr>
<td>F</td>
<td>1:10</td>
<td>200:1</td>
<td>100</td>
</tr>
<tr>
<td>Mean</td>
<td>1:10</td>
<td>33:1</td>
<td>98</td>
</tr>
</tbody>
</table>
Microfluidic Sperm Isolation Reduces DNA Fragmentation

Can Microfluidic Sorted Sperm Fertilize?

Microfluidic Sperm Sorter (MFSS) : Clinical Trial Ongoing (Japan)
### Microfluidic Sperm Sorter: Strengths and Weaknesses

<table>
<thead>
<tr>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ease of use / disposable</td>
<td>1. Only uses 40 μl semen - solution maybe multi-channels</td>
</tr>
<tr>
<td>2. No centrifugation needed - reduce physical or DNA damage</td>
<td>2. Efficiency hard to predict - under some circumstances unimportant</td>
</tr>
<tr>
<td>3. Toxicology testing - freestanding, no power source needed</td>
<td>3. Not compatible with current IVF insemination techniques</td>
</tr>
</tbody>
</table>

### Microfluidic Sperm Sorter: Final Thoughts

With respect to ART therapeutic use, the microfluidic sperm sorter by itself may seem unremarkable. The power of the device lies in its integration into a microfluidic system that is in development.

### Micro-insemination and Potential Use of Microfluidics in Embryo Development

- Revolutionized treatment of severe male factor infertility
- Invasive - 5-7% oocytes lysed - spindle damage and aneuploidy (?) - bypasses natural selection
- Long-term safety unknown
Micro-insemination Device

1. Removes randomness of sperm/egg interaction in conventional insemination
   - size constraint
   - direct delivery of sperm to egg
   - recirculation or re-insemination
2. Unlike ICSI, it is noninvasive
3. Potential integration with sperm sorter

Micro-insemination

<table>
<thead>
<tr>
<th>Insemination Concentration (x 10^6)</th>
<th>Center-well IVF</th>
<th>Microfluidic IVF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 0.5</td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>0.08 - 0.01</td>
<td>c</td>
<td>d</td>
</tr>
<tr>
<td>n=324</td>
<td>n=104</td>
<td>n=147</td>
</tr>
<tr>
<td>n=378</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

What is happening?
At high sperm concentration:
- high metabolic substrate usage
- concentrated degradative by-product
BAD for microfluidics

Why Might One Use Microfluidics in the Future?

1) Does something we cannot do today.
2) Does something we do today, but better.
3) Does something as well as we do today, yet less expensive.
4) Does something as well as we do today, yet less work.
5) Does something we do today, but safer.
Integration: ART Lab on a Chip (Smith et al., 2012)

Micro-insemination → Embryo Culture

Sperm Sorting

Embryo Analysis
- metabolic secretions
- genetic

Less Reagents/Cells
More Physiological
High Control/Automated

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Jun Ding, M.S.
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Coulter Foundation

References


Selecting spermatozoa; potential roles for electrophoresis and pharmacological enhancement

Laureate Professor R. John Aitken FRSE FAA
Priority Research Centre in Reproductive Science
Hunter Medical Research Institute
and University of Newcastle

Conflict of interest
RJA is associated with a commercial company, NuSep, which is involved in the commercialization of electrophoretic methods for the preparation of human spermatozoa.

Learning objectives

- Male infertility is a major cause of human infertility.
- The male factor involves not just a compromised ability to fertilize oocyte but also an impaired capacity to support normal embryonic development.
- Sperm preparation procedures should not only be rapid and efficient but should also select for cells exhibiting high levels of functionality and low levels of DNA damage. They should also eliminate leukocyte contamination.
- Several methods for sperm isolation exist including swim-up, discontinuous gradient density centrifugation and electrophoresis.
- Methods for pharmacologically enhancing the spermatozoa depend on a knowledge of the mechanisms responsible for defective sperm function.
- Oxidative stress is a major factor in the aetiology of defective sperm function and antioxidants have some potential in the preservation of these cells.

Clinical Problem

Contribution of ART to Australasian population

- Fertility in Australia is low, one in 4 women remaining childless at the end of their reproductive lives.
- One in 20 men are infertile.
- One in every 20 babies produced by ART.
- 70,541 ART treatment cycles reported in Australia and New Zealand in 2009, a 13.9% increase on 2008 and a 48.0% increase on 2005.
Male infertility is a major reason for ART

- In a vast majority of infertile males sufficient numbers of spermatozoa are present to achieve fertilization: it is sperm function that is compromised
- Normal male reproductive function is not just about achieving fertilization, it is also about supporting normal embryonic development.

DNA damage in the male germ line

Reproductive consequences of DNA damage in the male germ line

- Impaired fertilization (Benchaib et al., 2003; Virro et al., 2004; Aitken, 2004)
- Disrupted preimplantation development (Sakkas et al., 1998; Morris et al., 2002; Virro et al., 2004)
- Reduced pregnancy rates following natural or assisted conception (Coff, 2003; Duran, 2002; Burgum et al., 2004)
- Increased rates of abortion (Saleh et al., 2003; Carrell et al., 2003; Zini and Sigman, 2009)
- Increased rates of disease in children and young adults (Li et al., 1997; Aitken and Krausz, 2007; Aitken, 2004)

Select spermatozoa possessing low levels of DNA damage
The leukocyte problem

Leukocytes are powerful ROS producers

Log (1+x) Leucocytes/ml semen (10^4)

Log Luminol in Semen (counts/10 sec)

Aitken and Curry, 2011

Protection by Seminal Plasma

Washed sperm preparations

Seminal Plasma

Leukocytes @ 0.5 x 10^6 ml^-1

Aitken and Curry, 2011

Prediction of IVF Rates

Fitted Fertilization rate (%) vs. Fertilization rate (%) prediction

y = -4.668E-18 + 0.73

Aitken and Curry, 2011
Leucocytes (10^4 / ml) and CD45+ Dynabeads and Leukocyte removal from Sperm prep

Chemiluminescence (n 10^6 counts/5 min)

Oocyte Penetration (sperm/egg)

**Pre- Post-Dynabead

Aitken and Curry, 2011

Problems associated with Assisted Conception

- Incidence of stillbirth 4 times higher following IVF/ICSI than babies conceived naturally (Wisborg et al., 2010)
- Children conceived with ART also have about twice the risk of having a major birth defect or low birth weight than children conceived naturally (Hansen et al., 2002)

We should take the utmost care over the quality of the gametes we unite during IVF/ICSI.

Conventional methods of sperm isolation

Discontinuous density gradient centrifugation

- Percoll – PVP coated colloidal silicon
- Puresperm – Silane coated colloidal silicon
- Separation based on cell density

High density functional cells
- [good motility, good morphology, low levels of cell contamination]

Low density dysfunctional cells
- [poor motility, poor morphology, high levels of cell contamination]

Density gradient centrifugation

Pros: Improved yields Not dependent on sperm mobility

Cons: Time consuming Extraneous materials involved Increase in DNA damage Mechanical shearing forces
**Spermsep CS10 System Sperm Cell Separation**

Pros:
- Simple
- No contact with extraneous materials
- Rapid
- Effective with complex cellular mixtures
- Good yield
- Low levels of DNA damage

Cons: Limited field trial

---

**Electrophoretic sperm isolation**

- Graphs showing sperm recovery and DNA damage over different durations of electrophoresis.
- Data from Ainsworth et al., 2005:
  - Sperm recovery
  - DNA damage
  - Comparison of Original, Separated, and Residual samples.

---

**Electrophoretic isolation of spermatozoa**

- Graphs showing reduction in DNA damage after treatment with different methods.
- Data from Ainsworth et al., 2005:
  - Reduction in DNA damage over different samples.
  - Comparison of control and treated samples.
Electrophoretic isolation of spermatozoa

Leukocyte removal

Log leukocyte concentration/ml

Log chemiluminescence (cpm)

Susceptibility of spermatozoa to oxidative stress

ROS

High PUFA content

Limited Antioxidant Capacity

Lipid peroxidation

ROS and Spontaneous Pregnancy

Spontaneous pregnancy in 139 couples characterized by normal female partner

Redox activity (cpm x 10^4)

Cumulative pregnancy rate
TUNEL and 8OHdG

Application of Youden’s J to 8OHdG data

Increased oxidative DNA damage with silicon based density gradient methods

CS10 vs Percoll
Case Study

- Couple exhibiting long-term infertility (10+ years) associated with high levels of DNA damage in the male germ line. Patient produced an oligozoospermic ejaculate containing 3.2 million spermatozoa/ml and an equivalent number (2.1 million/ml) of contaminating round cells.
- Pre-separation:
  - 30% vitality
  - 12% motility
  - DNA damage - 26% TUNEL positive, 41% SCSA DFI
- Post-separation:
  - 62% vitality
  - 24% motility
  - DNA damage - 14% TUNEL positive, 15% SCSA DFI
- Intracytoplasmic sperm injection (ICSI) was conducted using the electrophoretically isolated spermatozoa.
- Oocytes were fertilized and normal blastocysts were generated after 5 days of culture. Transfer of two embryos was associated with the generation of a positive hCG signal followed by confirmation of a viable pregnancy by ultrasound.

This is the first clinical report of a viable pregnancy following the electrophoretic isolation of spermatozoa

Ainsworth et al, 2007

Preliminary Clinical trial

<table>
<thead>
<tr>
<th></th>
<th>Conventional</th>
<th>Electrophoretic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryo transfers</td>
<td>11</td>
<td>18</td>
</tr>
<tr>
<td>Pregnancies</td>
<td>2 (18%)</td>
<td>6 (33%)</td>
</tr>
</tbody>
</table>

When all embryos are derived from one sperm preparation method

Fleming et al., 2008

Ratio of X and Y bearing spermatozoa

Ainsworth et al, 2011
Mechanism – sialation high quality sperm

Preparation of spermatozoa for IVF

Electrophoretic Sperm Separation – cassette design
Pharmacological impacts on sperm function

Critical to appreciate mechanisms involved in causation of defective sperm function - particularly role of superoxide anion and hydrogen peroxide

Superoxide anion generation

Lipid peroxidation generates powerful electrophiles

4HNE drives mitochondrial ROS generation
Hydrogen peroxide mediates DNA damage

2H⁺ + 2O₂⁻ → O₂ + H₂O₂

Caspase activation

But

Impaired translocation of effectors of DNA cleavage such as Endo G, CAD and AIF

This is why all DNA damage in the germ line is oxidative

Impact of Vitamins E and C on DNA damage

Impact of penicillamine on sperm function

After 24 h incubation at 37°C
References -1

References -2

References -3
ESHRE 2012: Pre-congress course 2

Magnificent? High-power optical selection methods (IMSI vs ICSI)

Laura Rienzi
BSc MSc
Senior Clinical Embryologist
Laboratory

Learning objectives

1) Sperm selection procedures prior to ICSI
2) Clinical outcomes related to IMSI approach
3) Sperm phenotype and sperm quality
4) Evidences to conclude on this issue

I declare no commercial relationships or other activities that might be perceived as a potential conflict of interest

Male gamete abnormalities

Embryos developmental problem

Early events
- Failed fertilization
- Abnormal oocyte morphology
- Impaired cleavage

Late events
- Repeated implantation failures
- Increased pregnancy loss
Sperm DNA integrity

- TUNEL
- COMET

Sperm chromatin structural assay (SCSA)  Sperm chromatin dispersion (SCD) test

Sperm selection for ICSI

- SPERM BINDING ABILITY ASSESSMENT
- SPERM HEAD BIREFRINGENCE ASSESSMENT
- MAGNETIC-ACTIVATED CELL SORTING FOR SPERM PREPARATION
- REAL TIME FINE SPERM MORPHOLOGY ASSESSMENT

Sperm morphology and ICSI

Success rates of intracytoplasmatic sperm injection is independent of basic sperm parameters.

The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters.

The outcome of intracytoplasmic sperm injection is unrelated to 'strict criteria' sperm morphology

Peter Svalander1, Ann-Hekane Jakobsson, Ann-Sofie Forsberg, Anna-Carin Bengtsen and Mats Wikland
Sperm morphology and ICSI

The establishment of a pregnancy even with spermatozoa that are dysfunctional and with abnormal DNA may be attributed to the corrective role of selecting a single spermatozoon for ICSI.

Virro, Larson-Cook et al. 2004

Sperm morphology and ICSI

Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection.

Centre for Reproductive Medicine, University Hospital, Dutch-speaking Brussels Free University (Vrije Universiteit Brussel), Belgium.

Retrospective study

<table>
<thead>
<tr>
<th>Normal sperm morphology (ejaculated)</th>
<th>Abnormal sperm morphology (ejaculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of oocytes injected</td>
<td>4,406</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>72.5 ± 25.1</td>
</tr>
<tr>
<td>Embryo quality</td>
<td>73.6 ± 29.8</td>
</tr>
<tr>
<td>N° transfers</td>
<td>1226</td>
</tr>
<tr>
<td>Female age</td>
<td>34.1 ± 5.4</td>
</tr>
<tr>
<td>Pregnancy rate (%)</td>
<td>37.0</td>
</tr>
<tr>
<td>Clinical pregnancy rate (%)</td>
<td>33.0</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>19.0 ± 31.7</td>
</tr>
<tr>
<td>Live birth rate (%)</td>
<td>14.9 ± 28.4</td>
</tr>
</tbody>
</table>

* Significantly different

De Vos et al., 2003
Letter to New England Journal of Medicine:
"Selection of spermatozoa with normal nuclei to improve the pregnancy rate with
intracytoplasmic sperm injection"
Bartoov et al. (2001)

Introduction of a new concept to observe spermatozoa called 'motile-sperm organelle-
morphology examination' (MSOME) and to evaluate the fine nuclear morphology of motile
spermatozoa in real time.

Intracytoplasmic Morphologically Selected Sperm Injection
(IMSI)

IMSI: Sperm preparation
Bartoov et al., 2002
- Use of a density gradient in the preparation prior to selection
- Use of PVP (different concentration)
- Low temperature (according to sperm motility)
- Glass-bottom dish over the top of an 100x objective lens covered by a droplet of
  immersion oil
- Examination of individual spermatozoa at high magnification by the inverted microscope
  equipped with high-power Nomarski optics enhanced by digital imaging
- Sperm selection according to MSOME criteria
**IMSI: Sperm assessment**

Motile Sperm Organellar Morphology Examination

CRITERIA to select SPERMATOZOA SUITABLE for IMSI

The MSOME criteria for the morphological normalcy of the sperm nucleus were defined as:
- SMOOTH
- SYMMETRIC
- OVAL CONFIGURATION
- HOMOGENEITY OF THE NUCLEAR CHROMATIN MASS

(no more than one vacuole / less than 4% of the nuclear area)

The average length and width limits in 100 spermatozoa with a normally looking nucleus, are estimated as follow:
- LENGTH: 4.75 ± 0.28 µm
- WIDTH: 3.28 ± 0.20 µm

Bartoov et al., 2003

**IMSI: Sperm assessment**

Time expensive technique
- Highly trained embryologists required
- Additional cost to upgrade the equipment
Some studies have analyzed the impact of IVF-IMSI procedure on ICSI outcomes in terms of: fertilization rate, embryo development, pregnancy rate, implantation rate and abortion rate.

After 11 years is IMSI application based on clinical evidences?
Conclusions of the meta-analysis

- The current meta-analysis can conclude that IMSI not only significantly improves the percentage of top-quality embryos, implantation and pregnancy rates, but also significantly reduces miscarriage rates as compared with ICSI.

- However, a weakness of this meta-analysis is the variable study’s characteristic. Since the advent of IMSI, only one randomized controlled trial was published. Thus, to perform this meta-analysis, comparative studies in which IMSI cycles were matched with ICSI cycles also had to be included.

Source: Setti et al., 2010

IMSI: Prospective randomized study

168 Cycles

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>ICSI</th>
<th>IMSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age</td>
<td>28.80±4.28</td>
<td>29.67±4.32</td>
</tr>
<tr>
<td>Male age</td>
<td>32.53±4.87</td>
<td>33.97±5.52</td>
</tr>
<tr>
<td>Aetiology of infertility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male factor</td>
<td>39(48.1)</td>
<td>38(43.7)</td>
</tr>
<tr>
<td>Ovulatory</td>
<td>1(1.2)</td>
<td>2(2.3)</td>
</tr>
<tr>
<td>Tubal</td>
<td>10(12.3)</td>
<td>7(8.0)</td>
</tr>
<tr>
<td>Unexplained</td>
<td>24(29.6)</td>
<td>30(34.5)</td>
</tr>
<tr>
<td>Multiple factors</td>
<td>7(8.6)</td>
<td>10(11.5)</td>
</tr>
</tbody>
</table>

Source: Setti et al., 2010

Laboratory and clinical outcome

<table>
<thead>
<tr>
<th>Outcome</th>
<th>ICSI</th>
<th>IMSI</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of ICSI procedure (min)</td>
<td>13.55 ± 5.43</td>
<td>20.54 ± 9.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>80.97 ± 15.06</td>
<td>81.60 ± 10.55</td>
<td>NS</td>
</tr>
<tr>
<td>Grade 1 and 2 embryos on transfer day (%)</td>
<td>4.84 ± 6.93</td>
<td>5.01 ± 6.44</td>
<td>NS</td>
</tr>
<tr>
<td>Mean no. of embryos transferred</td>
<td>2.76 ± 0.46</td>
<td>2.72 ± 0.48</td>
<td>NS</td>
</tr>
<tr>
<td>Clinical pregnancy per initiated cycle (%)</td>
<td>16/81 (44.4)</td>
<td>47/87 (54.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Live birth rate per initiated cycle (%)</td>
<td>11/81 (13.5)</td>
<td>38/87 (43.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>42/215 (19.5)</td>
<td>66/228 (28.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Multiple pregnancy rate (%)</td>
<td>6/36 (16.7)</td>
<td>16/47 (34.0)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Source: Setti et al., 2010
Comparison of clinical pregnancy and implantation rates according to sperm characteristics

<table>
<thead>
<tr>
<th></th>
<th>ICSI</th>
<th>IMSI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No male factor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20/42 (47.6)</td>
<td>24/49 (49.0)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td><strong>Male factor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11/39 (28.2)</td>
<td>14/38 (36.8)</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td><strong>Sperm count</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 1 million/ml</td>
<td>4/16 (25.0)</td>
<td>4/16 (25.0)</td>
<td>ns</td>
</tr>
<tr>
<td>1-20 million/ml</td>
<td>7/22 (31.8)</td>
<td>10/27 (37.0)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Effects of advanced selection methods on sperm quality and ART outcome: a systematic review

- Most of the evidence provided regarding the advantages of using advanced sperm selection techniques remains to date preliminary in nature.
- Despite preliminary encouraging results, it should be noted that the numbers of patients assessed are limited, and most studies are underpowered to conclude on differences in pregnancy rates and live births.
- More research is needed to identify which infertility cases, if not all, will benefit from the application of these selection methods.

Said and Land, 2011

Which sperm phenotype does really reflect competence?
Does the presence of sperm nuclear vacuoles affect ICSI outcome?

<table>
<thead>
<tr>
<th></th>
<th>Experimental (n=28)</th>
<th>Control (n=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrieved oocytes</td>
<td>13.0 ± 5.0</td>
<td>12.1 ± 4.4</td>
</tr>
<tr>
<td>Injected oocytes</td>
<td>8.1 ± 3.6</td>
<td>8.4 ± 3.2</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>68.7 ± 10.5</td>
<td>72.8 ± 18.5</td>
</tr>
<tr>
<td>Top quality embryos (%)</td>
<td>23.0 ± 11.1</td>
<td>27.1 ± 29.4</td>
</tr>
<tr>
<td>Embryos transferred</td>
<td>3.0 ± 1.3</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Pregnancy rates (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abortion rate per pregnancy obtained</td>
<td>80%</td>
<td>7%*</td>
</tr>
</tbody>
</table>

Berkovitz et al. 2006

Does the presence of nuclear vacuoles influence the embryo’s competence to develop to the blastocyst stage?

<table>
<thead>
<tr>
<th></th>
<th>Grade 1/2</th>
<th>Grade 3/4</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of injected spermatozoa</td>
<td>18 (3.3 ± 0.3)</td>
<td>78 (12.2 ± 1.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Percentages (%) of embryos per injected spermatozoa</td>
<td>85 (3.3 ± 0.3)</td>
<td>85 (3.2 ± 0.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Day 4 blastocysts</td>
<td>88.4 (7.8)</td>
<td>82.3 (7.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Good quality day 4 embryos</td>
<td>69.3 (5.7)</td>
<td>60.9 (5.9)</td>
<td>NS</td>
</tr>
<tr>
<td>Blastocysts</td>
<td>66.5 (5.8)</td>
<td>58.6 (6.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Good quality blastocysts</td>
<td>67.3 (5.7)</td>
<td>55.1 (5.9)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Vandervelzen et al., 2008

Sperm morphology and IMSI and sperm quality

<table>
<thead>
<tr>
<th></th>
<th>150 sperm samples</th>
<th>100 sperm samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difference (%)</td>
<td>85 ± 2.9</td>
<td>64 ± 3.0</td>
</tr>
<tr>
<td>Acridine orange (%)</td>
<td>15.7 ± 6.1</td>
<td>12.4 ± 6.0</td>
</tr>
<tr>
<td>TUNEL (%)</td>
<td>14.0 ± 6.4</td>
<td>28.9 ± 12.7</td>
</tr>
<tr>
<td>Aneuploidies (%)</td>
<td>1.2 ± 0.4</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>

TD-testicular damage; PO=partial obstruction; A=A= 0.05 versus controls; B=B= 0.01 versus PO; C=C= 0.005 versus controls; D=D= 0.001 versus PO; E=E= 0.001 versus group A. Groallo et al., 2008
Nuclear vacuoles and sperm competence

- DNA Integrity
- Mitochondrial function
- Chromosomal aberrations

What Are Sperm Vacuoles?

Nuclear vacuoles are irregular entities in the condensed chromatin and are not limited by a membrane. Vacuoles occur commonly in human sperm nuclei but are generally observable only with TEM.

Are sperm vacuoles responsible for DNA damage?

[Images of sperm and vacuoles]

Palermo et al., 2011
Sperm DNA fragmentation

Basic sperm parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vacuolated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>63.5 ± 26.3</td>
<td>56.9 ± 1.7</td>
</tr>
<tr>
<td>Motility</td>
<td>56.9 ± 1.7</td>
<td>3.4 ± 1.2</td>
</tr>
<tr>
<td>Morphology</td>
<td>67%</td>
<td>67%</td>
</tr>
<tr>
<td>Vacuolation</td>
<td>23 (3.9)</td>
<td>22 (4.5)</td>
</tr>
</tbody>
</table>

Vacuolated Control

Total sperm: 576, 486
Fragmented (%): 23 (3.9), 22 (4.5)

Vacuolization: 67%

Palermo et al., 2011

Sperm DNA fragmentation

Basic sperm parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vacuolated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>79.5 ± 5.6</td>
<td>52.9 ± 5</td>
</tr>
<tr>
<td>Motility</td>
<td>52.9 ± 5</td>
<td>4.0 ± 2</td>
</tr>
<tr>
<td>Morphology</td>
<td>4.0 ± 2</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Vacuolation</td>
<td>68 (9.8)</td>
<td>61 (10.3)</td>
</tr>
</tbody>
</table>

Vacuolated Control

Total sperm: 697, 592
Fragmented (%): 68 (9.8), 61 (10.3)

Vacuolization: 65%

Palermo et al., 2011

Fluorescent in situ hybridization

Fixation

Decongestion

Hybridization

Palermo et al., 2011
Chromosomal content

[X, Y, 13, 14, 15, 16, 17, 18, 21, 22]

<table>
<thead>
<tr>
<th>Basic semen parameters</th>
<th>Concentration (x10^6/ml)</th>
<th>45.9 ± 1.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility (M% ± SD)</td>
<td>56.5 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>Normal (NM %)</td>
<td>42 ± 15</td>
<td></td>
</tr>
</tbody>
</table>

Vacuolated vs. Control

<table>
<thead>
<tr>
<th>Total sperm</th>
<th>Vacuolated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>623</td>
<td>10 (1.5)</td>
<td>7 (1.1)</td>
</tr>
</tbody>
</table>

Are we really speaking about vacuoles?

Surface "vacuole" or hollows on the surface

No relationship between chromosome aberrations and vacuole-like structures on human sperm head

Hollow Types

Hollows of sperm heads

Page 42 of 127
No relationship between chromosome aberrations and vacuole-like structures on human sperm head

Corrective role of the oocyte

An intact DNA does not appear to be a prerequisite for a successful fertilization as proven by the ability of ICSI, to yield satisfactory fertilization and pregnancy outcomes with compromised semen parameters, or immature spermatozoa such as those retrieved from the epididymis or resulting from a compromised spermatogenesis such as testicular specimens of non-obstructive azoospermic men.

These findings highlight the role of the oocyte as more than just a source of maternal DNA and nutrients to the presumptive conceptus, but also a contributing intricate machinery that can undo the damaging effects of a faulty male genome.
Lesson from IMSI approach (1)

Sperm quality may affect ICSI results in terms of embryo development (blastocyst formation) and clinical outcome.

No clear evidences have been published yet (evidence-based medicine, prospective randomized studies, enough power, identification of a specific category of patients) about the real efficacy of IMSI approach.

Lesson from IMSI approach (2)

Moreover contradictory results have been recently found from different groups about the significance and the nature of the presence of vacuoles on sperm competence.

The presence of sperm nuclear defects assessed by high magnification microscopy did not directly translate to chromosomal abnormalities or presence of DNA breakage.

We need to investigate better this aspect and try to find different aspects other than sperm morphology that can have an impact on ICSI outcome.

References

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- Svalander et al., 1996
- De Vos et al., 2003
- Vinro, et al. 2004
- Berkowitz et al. 2006
- Vanderzwalmen et al., 2008
- Garolla et al., 2008
- Watanabe et al., 2009
- Souza Setti et al., 2010
- Balaban et al., 2011
- Said and Land, 2011
- Palermo et al., 2011
Separating the wheat from the chaff.

Selection on the basis of sperm surface markers

Dr. Liliana Ramos, PhD

IVF laboratory, department of Reproductive Medicine, University Medical Centre Nijmegen, The Netherlands

Beyond IUI, IVF and ICSI - New developments in the selection and use of sperm for ART
Pre-congress course - Istanbul, Turkey 1 July 2012

I declare not to have commercial relationships or other activities that might be perceived as a potential conflict of interest.

Learning objectives:

• Sperm membrane: structure, receptors and apoptosis signals
• Surface markers: Phosphatidylserine (PS) and Hyaluronic Acid binding protein
• Magnetic cell separation (MACS): principles and uses
  • selection of non-apoptotic sperm
  • diagnostic value
  • ART outcome
• Hyaluronic acid (HA) binding test: principles and uses
  • selection for mature sperm
  • diagnostic value
  • ART outcome
Sperm selection: why?

There is an increasing need for non-invasive biochemical markers to select normal and functional sperm in ART, especially for ICSI.

Sperm selection for ART: how?

“Negative” selection (damaged sperm bind to membrane receptors; e.g. Annexin-V, FAS-ligands, TNF-receptors): unbound sperm can be collected and used for ART.

“Positive” selection (mature sperm are selected from damaged sperm; e.g. binding to HA-matrix): mature sperm is selected for ART; unbound/unselected sperm is discarded.

Sperm membranes are highly polyunsaturated; they have a specific constitution and function: changes are necessary for acrosom reaction, ZP recognition and oocyte for fusion.
“Negative” markers and sperm selection:
principles and theory of the Annexine V binding test

Annexine V-binding (membrane changes upon external damaging substances like ROS, UV- and Y-radiation). Externalization of PS from the inner to outer membrane layer.

Damage induced by ROS

Magnetic microbeads are conjugated with Annexine V: damaged sperm binds to Annexine V and are retained in a magnetic field.
I. MACS –Annexine V for diagnostic testing (density gradient centrifugation and MACS)

Apoptotic markers: activated Caspase 3 / mitochondrial membrane potential (MMP) / TUNEL
Sperm fertilization potential in hamster oocyte penetration and hamster ICSI-test

MACS-Annexine V negative sperm:
- higher % motile sperm
- lower % sperm apoptotic markers
- higher % oocyte penetration
- no difference in chromatine decondensation after ICSI


II. MACS –Annexine V for diagnostic testing (density gradient centrifugation and MACS)

Patients: 60 couples with unexplained infertility and failed IUI

Increased % of sperm with positive hemizona assay.
Conclusion: possible benefit of MACS in unexplained fertility.
No clinical outcome described


Clinical uses and limitations

- Minimal amount of sperm for effectively recovery with MACS-Annexine V?
- Not clear whether suitable for IUI / IVF or only ICSI
- Contamination and recovery rates?
- No clinical uses at present
Hyaluronic acid is a polysaccharide of the glycosaminoglycans class.


G. Huszar (University of Yale) developed hyaluronic acid (HA) binding test.

"Positive" markers and sperm selection: principles and theory of the Hyaluronic acid (HA) binding test

A physiologic marker is the binding capacity of sperm to hyaluronic acid (HA), an extracellular matrix component secreted by cumulus cells.

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Evaluation of HA-binding and sperm integrity

HBA-slides (provided by MidAtlantic Diagnostics)
25 Semen samples:
12 normospermia
13 abnormal (OAT or combinations)

%HA binding = \# motile bound sperm \times 100
\# total motile sperm

Tests
TUNEL
CMA3
* original sample
* sperm fraction after gradient centrifugation
* HA-bound fraction

Results (1)
% HA-binding and semen parameters

Pearson correlations
- Concentration: \( r = 0.551 \) P=0.006
- % Morphology: \( r = 0.482 \) P=0.015
- % Motility: \( r = 0.685 \) P<0.001

Prognostic value % HA-binding test for normospermia (ROC analysis)
AUC = 0.891 P=0.001
Cut-off value: 45% binding (75% and 92% sensitivity and specificity resp.)

Results
% TUNEL and CMA3 positive sperm in each sperm fraction
Zeta and HA-binding: evaluation of sperm integrity


Semen sample 77 patients

CMA3 (Protamine content improvement with both Zeta and HA-binding)

Sperm chromatin dispersion test (SCD) (DNA integrity improved, Zeta method better)

Papanicolau stain (improvement sperm morphology with both methods)

Diagram for sperm selection using zeta potential. Sperm suspension is pipetted into a positively charged tube (10-50% charge). Sperm with a zeta potential lower than critical point are adhered upon the walls of the tube and are discarded.

HA-bound sperm: DNA damage evaluated by acridine orange fluorescence (AOF)

Yagci et al. J. Andrology 2010. 31(6):566-572

Green fluorescence (double-strands DNA)
- Total fraction: 54%
- HA-bound sperm (in spot): 99%

Morphology of HA-bound sperm evaluated by strict criteria

- The proportion of normal spermatozoa was higher in HA-bound with a 3.04-fold improvement (95% confidence limits: 1.9-4.7) in 37 teratozoospermic men.
HA-bound sperm and morphology with MSOME evaluation

6.592 sperm/56 patients
5579 HA-bound sperm
2.7% presented normal morphology
No statistical difference in morphology between bound/not bound sperm

Petersen et al. Reproductive Biology and Endocrinology 2010 8:149

HA-bound sperm and aneuploidy
HA-selected spermatozoa reduced 4- to 6 fold the frequency of chromosomal aneuploidy


Conclusions diagnostic test
The HA-binding test is a promising diagnostic tool in assessing fertility potential in sperm samples
Sperm with the capacity to bind to a monolayer of hyaluronic acid present low percentage of DNA-damage and higher chromatin condensation
I. HA and ART outcome: prospective study
Nijs et al. Andrologia 2010 42(5):29-6

68 patients: ½ IVF and ½ ICSI (evaluation of HA-binding in neat sample)
- Semen analysis: HA-binding not correlated to morphology, concentration or motility
- % fertilization: HA-binding not predictable for fertilization failure
- Embryo quality: correlation with HA-binding
- Ongoing pregnancy or Cumulative pregnancy rate: no correlation with HA-binding

Limited predictive value/ limited clinical use

II. HA and ART outcome: prospective, blinded controlled trial

Patients with unexplained infertility: ½ IVF and ½ ICSI
(evaluation of HA-binding in neat sample)
- HA-binding cut-off: < 60%, 60-80%, >80%
- % fertilization: HA-binding not predictable for IVF fertilization
- Embryo quality: similar in all cut-off groups

Limited predictive value/ limited clinical use
III. HA and ICSI outcome: prospective randomized study


44 Patients: ICSI with ½ HA-bound sperm (HA+) ½ unbound sperm (HA-)
- % fertilization: HA(+) 75% HA(-) 70%
- Zygote score, embryo quality and # 4c- embryos: similar in both groups

HA(+) higher fertilization rate; pregnancy rate not different

Limited predictive value/ limited clinical use

IV. HA and ART outcome: freezability of sperm


Sperm donors (129): semen analysis and % HA-bound sperm

Predictive value HA-binding significant, but not better than % motility after 1- to 4 hours

Limited predictive value/ limited clinical use

V. HA and IVF outcome


175 IVF patients: 3 or > oocytes.
HA-binding test in an aliquot semen

HA-binding correlates with motility and morphology
Poor predictive value of HA-binding for poor fertilization

Limited predictive value/ limited clinical use
VI. HA and IVF outcome: retrospective study
(20 samples, T1: raw sample, T2: the time of insemination).

No correlation for %HA-binding in the raw sample or after gradient centrifugation (at the time of insemination).
No correlation for fertilization rates.

VII. HA and IVF outcome (under Italian law)
60 IVF patients
HA-binding test in an aliquot semen, TUNEL

No relationship between HA-binding with fertilization, cleavage, embryo quality, clinical pregnancy, miscarriages.

Limited predictive value/ limited clinical use.

VIII. HA and ICSI outcome (under Italian law)
1) 293 ICSI couples HA-selected vs 86 standard ICSI
HA-binding test in an aliquot semen, TUNEL
2) 206 ICSI couples

Conclusion both studies: HA-bound sperm significantly improved embryo quality and implantation.

Predictive clinical value.
HA- clinical uses and limitations

- No clear beneficial outcome after injection of HA-bound sperm compared to standard ICSI
- Only (highly) motile sperm can bind to HA- is this parameter sufficient to select best sperm?
- Only 2 Italian papers found a positive correlation between PICSI and ART outcome: effect of oocyte selection?

Conclusions and discussion

- Potentially role for selection based on other membrane markers like fertilin alpha (ADAM-1), beta (ADAM-2) or ADAM-3/ infertility-associated sperm protein (IASP) / Zeta-binding protein / 57 kDa protein
- Role of Hyaluronan binding protein-1 (HABP-1)
- Low clinical application of HA-binding test and PICSI, potential bias?
- No randomized clinical trials (RCT) on HA-bound sperm outcome: time to proceed?
- Both systems (MACS/HA) only suitable for samples with relative high % motile sperm, not suitable for extreme OAT/ PESA/ TESE

References list


May the force be with you

Using immotile sperm in ART

Verheyen Greta, PhD
Centre for Reproductive Medicine – UZBrussel – Brussels – Belgium
ESHRE 2012 Istanbul

Disclosure

I declare to have
no commercial relationships
no conflict of interest

Learning objectives

- To understand the origin of sperm motility
- To understand the importance of sperm motility for fertilization and outcome of ART
- To know the different techniques/procedures to distinguish immotile vital and dead sperm
- To know the results of ICSI with immotile sperm
- To know the success rates of ICSI with immotile sperm
- To have an overview of the literature
Introduction

Motility

One of the main characteristics of spermatozoa

\[ \downarrow \]

Natural conception

Migration vagina \( \rightarrow \) Fallopian tubes
Penetration of cumulus complex
Binding to zona pellucida

Introduction

Origin of motility

- Sperm ultrastructure
  - Spermatogenic cells: centrosome
  - Spermiogenesis: tail formation
- Energy source
  - Spermatogenesis: mitochondria
  - Spermiogenesis: midpiece (mitochondria)
- Sperm maturation
  - Epididymal transit
  - Acquire motility

Sperm ultrastructure

Centrosome = microtubule organizing centre (MTOC)
Sperm ultrastructure

Centrosome characteristics
- composed of two centrioles
  - perpendicular to each other
  - surrounded by protein mass
- MTOC: production of microtubules (cell cytoskeleton)
- Role in mitosis: spindle formation
- Formation of cilia and flagella

sperm flagellum
- Specialized structure

Sperm ultrastructure

- Role of the sperm centosome
  - Proximal centriole → sperm aster after fertilization
    → spindle formation (mitosis)
  - Distal centriole → tail during spermiogenesis

Sperm ultrastructure

Axonemal structure
Sperm ultrastructure

Energy metabolism

- Mitochondria play a key role in cell metabolism
- Motor for sperm motility
- ATP production

Sperm maturation

- Passage through epididymis
- Sperm acquire the ability for progressive motility
Diagnostic semen analysis

- Three basic semen parameters
  - Sperm concentration
  - Sperm morphology
  - Sperm motility
    - Four categories A-B-C-D
    - WHO criteria 2010: Normal value ≥35% progressive motility A+B
  - Sperm vitality
    - If <40% progressive motile sperm
    - Eosin-nigrosin test

- Discriminate absolute and virtual asthenozoospermia
  - Centrifugation of semen
  - Extensive search

- Discriminate absolute asthenozoospermia and necrozoospermia
  - Viability test
  - Integrity of sperm membrane
  - Dye exclusion tests
    - eosin-nigrosin
    - eosin Y test

Asthenozoospermia

- Criteria
  - Asthenozoospermia: <35% A+B
  - Severe asthenozoospermia: <5% A+B+C
  - Absolute asthenozoospermia: 100% D

- Diagnosis of absolute asthenozoospermia

- Prevalence of absolute asthenozoospermia: 1:5000 men Eklsson et al. 1977
Absolute asthenozoospermia

- Aetiology?
  - Ultrastructural defects
    - Congenital
    - Inherited
  - Necrozoospermia
    - Genital infection
    - Oxidative stress
    - Cryopreservation
    - ASA
    - Metabolic disorders
    - Exposure to environmental pollutants
  - Prolonged period of anejaculation
  - Unexplained

Ultrastructural defects

- Defect in spermiogenesis
  - Defective axonemal structure
  - >200 genes involved in microtubule synthesis (Yatsenko et al., 2010)
  - Genetic origin
- Immotile-cilia syndrome (Afzelius 1976)
  - Autosomal recessive disorder
  - Absence of dynein arms
  - Prevalence 1:20000 live births (Cayan et al. 2001)
  - Kartagener syndrome: combined with situs inversus
    - Dysfunction of tracheobronchial cilia → bronchitis/sinusitis
  - Mostly permanent condition

Ultrastructural defects

Transmission electron microscopy

Normal axoneme

Ortega et al., 2011
Ultrastructural defects

Transmission electron microscopy

Absence of dynein arms

Absence of central microtubuli

(9+0)

Ortega et al. 2011

Necrozoospermia

- Rare condition (0.2-0.5% of infertile men)
- Origin in testis or epididymis
- Accurate clinical assessment
  - Medical history, urogenital examination, hormone profile, semen and urine culture, seminal biochemistry, transrectal ultrasonography, testicular biopsy
  - Lecomte et al. 1998, Tournaye et al., 1998
- Identify the origin and correct if possible
- Not always permanent condition
- Role of testis biopsy
  - Tournaye et al. 1996

ICSI and absolute asthenozoospermia

- ICSI = only possible treatment option
  - High rate of fertilization failure (Liu et al. 1995)
  - Decreased fertilization and pregnancy rates (Nagy et al. 1995)
ICSI and absolute asthenozoospermia (AA)

- May be reversible  Vandervorst et al. 1997
  11 couples, 11 first cycles with AA  → 12.4% FR  → 0% PR

  9 couples, 16 subsequent cycles
  - 12 cycles with motile sperm  → 56.5% FR  → 4 pregnancies
  - 4 cycles with immotile sperm  → 15.6% FR  → 0 pregnancies

Absolute asthenozoospermia

- If no corrective action
- If only immotile sperm available

  The lab must solve the problem
  Challenge = discriminate immotile live sperm from immotile dead sperm

Absolute asthenozoospermia

- 100% immotility in the ejaculate
- Two possibilities
  - Viability – 0%  ⇒  necrozoospermia
  - Viability > 0%
    - How to select viable sperm for ICSI?
    - Exposure to dyes
      Unsuitable for ICSI
Necrozoospermia / low viability rate

- Second semen sample
- Go to the testis
  - Increased chance to find live/motile sperm
  - No guarantee
  - Overnight incubation ≠ valid option
    - May improve the quality of motility
    - No effect on viability

Sperm available for ICSI

- Three conditions
  - Sufficient motile sperm available to inject all oocytes
    → Abundantly available ⇔ after extensive search (NOA)
  - Only immotile sperm available
    → Rare condition
  - Insufficient motile sperm available to inject all oocytes
    → Occurs regularly
    → First oocytes injected with motile; others with immotile sperm

Immotile live ⇔ immotile dead?

Available methods:

1. Hypo-osmotic swelling test (HOST)
2. Exposure to motility enhancers (ME)
3. Mechanical touch technique (MTT)
4. Laser-assisted immotile sperm selection (LAISS)
5. Birefringence polarisation microscopy (BPM)

Reviewed by Ortega et al. 2011
1. Hypo-osmotic swelling test

- First described by Jeyendran et al. 1984
- Principle: test the functional integrity of sperm
  - Dye exclusion tests: structural integrity
- Principle based on osmosis
  - Live sperm swell/curl in a hypo-osmotic condition
  - Dead sperm remain unchanged in a hypo-osmotic condition
- Patterns:

Zeyneloglu et al. 2000

1. Hypo-osmotic swelling test

- Jeyendran medium
  - Fructose - Na citrate - water (155 mosmol/kg)
- Toxic for use in ICSI?
- Comparison HOS media in sperm-survival test
  - Verheyen et al. 1997
    - Earle's buffer - milli-Q water 1:1 (139 mosmol/kg)
    - Better survival than in Jeyendran medium
- Procedure
  - Exposure for 1 min
  - Re-equilibration in isotonic medium before injection
1. Hypo–osmotic swelling test

- ICSI results
  - Casper et al. 1996 (8 cycles)
    - Fertilization: 43% with HOS-selected sperm
    - Embryo development: 39% with HOS-selected sperm
  - Barros et al. 1997 (cycles)
    - 41.9% fertilization
    - 2 clinical pregnancies
- Applied in several centres
- Interpretation not always clear
  - Especially in frozen sperm

2. Exposure to motility enhancers

- Phosphodiesterase inhibitors (Task and Means, 1983)
  - Increase intracellular cAMP
  - Enhance sperm motility
- Pentoxifylline (PTX)
  - First described by Yovich et al. 1988
  - Embryo toxicity in mouse
    - Tournaye et al. 1993
    - No negative effect if only sperm is exposed
      - Tournaye et al. 1994; Terriou et al. 2000

2. Exposure to motility enhancers

- ICSI with immotile testicular sperm
  - Kovacic et al. 2006

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>+ PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>Search time/cycle (min)</td>
<td>120</td>
<td>30</td>
</tr>
<tr>
<td>Cycles with motile sperm</td>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>50.9a</td>
<td>66.0a</td>
</tr>
<tr>
<td>Clin. pregnancy rate (%)</td>
<td>26.7</td>
<td>58.3</td>
</tr>
</tbody>
</table>
2. Exposure to motility enhancers

- Comparison HOST and PTX on immotile testicular sperm
  Mangoli et al. 2011

<table>
<thead>
<tr>
<th></th>
<th>HOST</th>
<th>PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>MII oocytes</td>
<td>336</td>
<td>311</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>41.1*</td>
<td>62.1*</td>
</tr>
<tr>
<td>Cleavage rate (%)</td>
<td>86.2</td>
<td>89.1</td>
</tr>
<tr>
<td>Clin. pregnancy rate (%)</td>
<td>16b</td>
<td>32b</td>
</tr>
</tbody>
</table>

2. Exposure to motility enhancers

- Limited application in clinical practice
- May induce motility in vital immotile "testicular" sperm
- Mostly applied on testicular sperm
- Effectiveness on ejaculated immotile sperm ???
- UZ Brussel: not routinely applied in clinical practice

3. Mechanical touch technique

- First described by Soares et al. 2003
- "Sperm tail flexibility test"
- Principle: test the flexibility of the tail
  - Live sperm: flexible tail - recovers initial position
  - Dead sperm: rigid tail
- ICSI results
  - Soares et al. 2003 33.3% fertilization rate (ejaculated sperm)
  - de Oliveira et al. 2004 73.4% fertilization rate (fresh testicular sperm)
- Expertise of the embryologist
- Applied in UZ Brussel since many years
4. Laser-assisted selection (LAISS)

- First described by Aktan et al. 2004
- Principle: single laser shot close to the tail tip
  - Live sperm react by tail curling
  - Dead sperm show no reaction
- Advantages
  - No exposure to non-physiological media or toxic compounds
  - Rapid procedure
  - Easy interpretation
- Disadvantage
  - Expensive equipment (laser)

4. Laser-assisted selection (LAISS)

- Risks?
  - No damage, permeabilization of sperm membrane
    - Montag et al. 1999
  - No increase in DNA fragmentation
    - Montag and Rink 2001
- ICSI results
  - Higher fertilization and cleavage rate than MOST
    - Aktan et al. 2004
    - Fertilization rate: 64.2% vs 46.5%
    - Cleavage rate: 79.6% vs 51.4%
- Clinically applied in UZ Brussel since 2010
- Not widely applied (expensive)
Comparison MTT and LAISS in UZ Brussel

- Unpublished results
- Consecutive periods
  - MTT: Jan 2009 - Jan 2010 23 cycles
  - LAISS: Jan 2010 - Dec 2011 29 cycles
- Immotile sperm only used for ICSI after extensive search for motile spermatozoa
- Sperm origin
  - Fresh or frozen ejaculated sperm
  - Fresh or frozen testicular sperm
  - Frozen electro-ejaculated sperm

<table>
<thead>
<tr>
<th></th>
<th>MTT</th>
<th>LAISS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>Oocytes injected</td>
<td>143</td>
<td>248</td>
</tr>
<tr>
<td>Motile MTT</td>
<td>49</td>
<td>94</td>
</tr>
<tr>
<td>Motile LAISS</td>
<td>103</td>
<td>135</td>
</tr>
<tr>
<td>Immotile MTT</td>
<td>94</td>
<td>185</td>
</tr>
<tr>
<td>Immotile LAISS</td>
<td>135</td>
<td>10</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>(46.9)</td>
<td>(31.9)</td>
</tr>
<tr>
<td></td>
<td>(45.6)</td>
<td>(28.9\*)</td>
</tr>
<tr>
<td>Embryo transfer</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td>+ hCG</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>3 cycles (2 children)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 cycles (2 children)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 cycle (0 children)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* \(P=0.008\)

Conclusion
- No difference in effectiveness between MTT and LAISS
- Weakness of the study
  - Consecutive periods
  - Low number of cycles
- Need for prospective randomized trial
  - Few cycles with absolute asthenozoospermia included
  - Rare condition.
5. Birefringence–polarisation microscopy

- Principle: decomposition of a ray of light into two rays when it passes the sperm head

- First described for human sperm by Baccetti et al. 2004
  - Live sperm are birefringent
  - Dead sperm are not birefringent

---

5. Birefringence–polarisation microscopy

**Gianaroli et al. 2008**

- Birefringent characteristics of sperm
  - Well-organized and compact structure
  - Birefringent nucleus, acrosome, midpiece, tail
  - Longitudinal orientation of protein filaments
  - Confirmed by transmission electron microscopy

- Birefringence as new criterion for sperm selection
  - ICSI with selected birefringent sperm in cycles with severe OAT with no 'progressive' motility + TESE cycles

<table>
<thead>
<tr>
<th>Control</th>
<th>Birefringence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycles</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>Ongoing PR per ET</td>
<td>8</td>
<td>23</td>
</tr>
</tbody>
</table>

---

5. Birefringence–polarisation microscopy

- Partial sperm head birefringence
  - Indicates acrosome reaction
  - Higher clin PR and IR compared with non-reacted sperm

**Gianaroli et al. 2010**

- Comparison ICSI with birefringent-selected sperm or HOS–selected sperm in absolute asthenozoospermia

<table>
<thead>
<tr>
<th>Cycles</th>
<th>HOST</th>
<th>Birefringence</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilization rate</td>
<td>61.3</td>
<td>76.8</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Cleavage rate</td>
<td>51.2</td>
<td>68.7</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Clin pregnancy rate</td>
<td>11.1</td>
<td>45.0</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>
5. Birefringence-polarisation microscopy

- Partial sperm head birefringence
  - Indicates acrosome reaction
  - Higher clin PR and IR compared with non-reacted sperm
    Gianaroli et al. 2010
  - No comparative studies available
  - 46% pregnancy rate
    Ghosh et al. 2012
- Limited clinical application
  - Promising but expensive
  - Not well-known

ICSI with immotile sperm

- First pregnancy reported in 1997
  Kahraman et al. 1997
- Literature reports large ranges
  - Fertilization rate: range 3% - 76.4%
  - Pregnancy rate: range 0% - 38%
- Large series reported
  Kovacic et al. 2006
  - 47 cycles with immotile testicular sperm
  - 66% fertilization rate
  - 38.3% pregnancy rate

ICSI with immotile sperm

- Discrepancies in literature may be caused by
  - Overall time/effort spend for searching motile sperm
  - Real immotility vs virtual immotility of individual spermatozoa
  - Accurate observation of individual sperm
  - Choice of the technique to select viable sperm
  - Expertise of the embryologist with each of the techniques
  - Ejaculated sperm versus testicular sperm

- Search/observation time ↑
  - Real Immotility ↑
  - Fertilization rate ↓
Conclusions

- Absolute asthenozoospermia = rare condition (1/5000)
- Different techniques to distinguish viable and non-viable immotile spermatozoa
  - Complexity, reliability
  - Time, cost
  - Pros and Cons
- No prospective randomized trial available in literature
  - Limited comparative studies
  - Limited number of cycles
  - Mixed motile/immotile sperm for ICSI within a cycle
- Success rate with immotile sperm remains lower than with motile sperm

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No sperm today.
Unexpected azoospermia at OPU

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Assaf Harofeh Medical Center
Tel Aviv University, Israel

Disclosure

Herewith I declare having no commercial relationships or other activities that might be perceived as a potential conflict of interest.

Learning objectives:

- To arrive to the correct diagnosis of crypto-azoospermia/azoospermia.
- To treat unexpected azoospermia according to stepwise paradigm.
- To use preventive measurements to minimize the occurrence of unexpected azoospermia on OPU day.
Definition of Azoospermia

Absence of sperm on standard microscopic examination

Meaning:
Search for sperm cells at 400X magnification in a sample of the pellet of semen following a 15min centrifugation at 3000 g

WHO 1999

Definition of Aspermia

Failure of formation or emission of sperm

Azoospermia - Practical definition in IVF laboratories

Absence of sperm after a meticulous search in droplets containing the whole pellet sample

Search for sperm in an Extended Sperm Preparation – ESP

ESP –
Distributing aliquots from the pellet into 20 to 25 droplets of 25µL (pulling out about 12 µL to flatten the droplet).

Ron-El et al., 1997

With the ESP method, one can detect cases of:

Cryptozoospermia, Quasi azoospermia

Intermittent Azoospermia - occurs mainly in cases of re-canalization or reversal after vasectomy

Sperm Concentration - Definitions

Normal concentration, Lower limit

Oligozoospermia

Severe Oligozoospermia

One or few sperm in the examination chamber

One or few sperm in the ESP droplets

Normal concentration, Upper limit
Unexpected Azoospermia at OPU

Will appear more often in:

1. crypto azoospermia cases
2. Reversal of vasectomy

Meniru et al, 1997

Unexpected Aspermia at OPU

May appear unrelated to sperm characteristics

The occurrence of unexpected aspermia

Rare event. Less than 0.5%
Assaf Harofeh, TAU

The occurrence of unexpected azoospermia

Rare event. About 0.5%
Assaf Harofeh, TAU

Number of treated cycles in Assaf Harofeh, TAU is 1100-1200 per year

Approaches to overcome the problem

In cases of aspermia

1. Repeated sperm emission at the clinic
2. Repeated sperm emission outside the clinic (home, hotel)
3. Repeated sperm emission by coitus – using a medical condom
   a condom without spermicidal agent
4. Use of Sildenafil (Viagra) in case of erectile dysfunction

Time frame:
1-9 hours post OPU time.
This is the interval period for insemination without compromising the oocyte quality, e.g. fertilization rate and embryo quality are unchanged
Fisch et al, 1989
Cases of aspermia

Preventive measurements

1. Inquiring the patient, at admission, about difficulties to produce sperm
2. Storage of frozen sperm prior to IVF treatment

Approaches to overcome the problem

In cases of azoospermia

1. Repeated sperm emission at the clinic
2. Repeated sperm emission outside the clinic (home, hotel)
3. Repeated sperm emission by coitus – using a medical condom
   a condom without spermicidal agents
4. Emergency testicular aspiration (PESA) or biopsy (TESE)

Cases of azoopermia

Preventive measurements

1. Increasing abstinence period to 5 days
2. Storage of frozen sperm prior to IVF treatment

Some of these approaches and measurements do not coincide with data appearing in the literature
### Sperm density – Sperm Concentration - some facts

Ideal semen volume and sperm density are achieved after 2-3 days abstinence period

**Shorter abstinence** periods decrease sperm density

Tyler et al., 1982; Nnatu et al., 1991

**Longer abstinence** periods increase sperm density, but also increases the proportion of dead, immobile or morphologically abnormal sperm

Pellestor et al., 1994

The highest concentration of sperm is in the initial portion of the ejaculate

---

### Daily practice to solve unexpected azoospermia –

Albeit the different knowledge in the literature

1. **Repeated Ejaculation.**
   - especially in the cryptozoospermia group, a repeated ejaculation may produce better quality of ejaculate.
   - In our experience, about 30% will have better quality in their repeated sperm sample than in the first one.

2. **Immediate TESE if no sperm is present**,
   - also not in the repeated ejaculate

   Since 2007 we had 8 patients with no visible sperm in their first and second ESP on which urgent TESE was performed. Sperm was found in 4 of them.

---

In a study where 3 cases were taken to Urgent TESE only in one of them sperm were detected.

Song SJ et al., 2010

Meaning,

Urgent TESE is not a treatment with which sperm presence is guarantied, also not in crypto azoospermia.
The probability cryptoazoospermic to turn into azoospermia

Out of 39 patients with severe non obstructive azoospermia:
- 5 x 10^6/mL

In a 42 months follow up:
- 7 (18%) became crypto-azoospermic
  - average count 0.1 x 10^6/mL
- 5 (13%) became azoospermic
  - was confirmed in ≥ 2 centrifuged specimens

Song SH et al., 2010

---

**Unexpected azoospermia at OPU – diagnosis and treatment**

- ESP – search in droplets
  - Sperm found
  - Motile sperm
  - Immotile sperm
  - No sperm

- Repeat ejaculation
  - Sperm found
  - Motile sperm
  - Immotile sperm
  - No sperm

- Urgent TESE in cases of NOA
  - Urgent PESA, TESA

- ICSI
  - Sperm found
  - Motile sperm
  - Immotile sperm
  - No sperm
  - Sperm donation
  - IVF

---

**Conclusions:**

- Unexpected azoospermia on OPU day is a rare occurrence
- Diagnosis should be confirmed by ESP
- Repeated ejaculation may solve the problem
- Urgent PESA/TESE or TESE should be performed when no sperm was detected also in the repeated ejaculate.
  - Patients should know that this procedure may produce sperm only in part of the cases.
- Preventive measurements should be offered to the patient when probability of unexpected azoospermia exists.
  - Backup of frozen husband sperm or donor sperm should be suggested to the couple
The delay in the use of semen sample for insemination may create DNA fragments.

Although DNA fragments may be present, they have no effect on pregnancy rates.

References

"The beauty and the beast" when the sperm fails to activate the oocyte: what’s next?

Ragaa Mansour, M.D., Ph.D.
Director, The Egyptian IVF-ET Center
EHSRE 2012

Disclosure

Ragaa Mansour, M.D., Ph.D.
No thing to disclose

Learning Objectives

1. Estimate cases of total failure of fertilization in IVF/ICSI
2. Discuss causes of fertilization failure in IVF/ICSI
3. Describe various measures to improve fertilization
Intracytoplasmic sperm injection (ICSI) has become the most effective therapeutic treatment for male factor infertility

Devroey P et al., 2004

However, total failure of fertilization still occurs in some cases, such as globozoospermia, teratozoospermia, immotile spermatozoa, and even unexplained cases

Rybouchkin AV et al., 1997; Plachot M et al., 2002; Khalil PE et al., 2003; Fischel S et al., 2000

It is estimated that failed fertilization occurs in 2% – 3% of ICSI cycles

Ebner T et al., 2004; Makute NG et al., 2003; Hantla RS et al., 2003.
Fertilization rate after ICSI

1- Oocyte "the beauty" and sperm "the beast" quality.
2- ICSI technique itself.
3- Tissue culture conditions in the IVF lab.

Improving fertilization after ICSI

1. The impact of spermatozoa preincubation time and spontaneous acrosome reaction on ICSI.
2. Electrical activation of oocytes after ICSI.

Manouz et al., 2008, Manouz et al., 2009

Many hours elapse between ejaculation and in vivo fertilization. During this time, sperm capacitation and the acrosome reaction occur. These crucial steps in gamete interaction allow the penetration of the zona pellucida and fusion with the oocyte membrane.

Wassarman PM. exocytosis, and fusion. Cell. 1999
The ability of spermatozoa to undergo a normal acrosome reaction and the rate of this reaction, are important indicators of fertilizing ability.

With (ICSI), zona pellucida penetration and oolemmal fusion are bypassed and the acrosome reaction may be seen as unnecessary.

However, the introduction of an acrosome intact sperm into the ooplasm by ICSI seems to physically disturb sperm chromatin decondensation.

Induction of an artificial acrosome reaction

increased fertilization rates and accelerated pronucleus formation
Hypothesis
Based on the observation that the acrosome reaction occurs spontaneously during incubation in a defined medium and is time dependent, we hypothesized that extending the preincubation time of spermatozoa might improve the fertilization rate in ICSI.

The aim of the study
To correlate the acrosomal status of the spermatozoa at the time of ICSI and the fertilization rate, and determine the optimum time interval between semen processing and incubation before ICSI.

Design
Semen Processing

<table>
<thead>
<tr>
<th>Sperm incubation at 5% CO₂ and 37°C for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM  a) one hour  ICSI</td>
</tr>
<tr>
<td>EM  b) Three hours  ICSI</td>
</tr>
<tr>
<td>EM  c) Five hours  ICSI</td>
</tr>
</tbody>
</table>
Sibling oocytes from each patient were allocated to one of the three study groups according to sperm incubation time:

- a- one hour
- b- Three hours
- c- Five hours

Table 1. Outcome of intracytoplasmic sperm injection (ICSI) according to different incubation times of spermatozoa

<table>
<thead>
<tr>
<th>Sperm incubation time before ICSI</th>
<th>1 h group</th>
<th>3 h group</th>
<th>5 h group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age year (mean ± SD )</td>
<td>32.14 ± 3.52</td>
<td>32.03 ± 3.58</td>
<td>33.01 ± 1.54</td>
</tr>
<tr>
<td>Oocytes retrieved (mean ± SD )</td>
<td>310 (9.9 ± 2.1)</td>
<td>340 (10.2 ± 2.5)</td>
<td>705 (10.04 ± 1.55)</td>
</tr>
<tr>
<td>2PN oocytes (mean ± SD )</td>
<td>446 (8.5 ± 4.1)</td>
<td>672 (7.9 ± 2.5)</td>
<td>640 (8.2 ± 2.3)</td>
</tr>
<tr>
<td>Eggs retrieved (mean ± SD )</td>
<td>435 (6.0 ± 0.1)</td>
<td>498 (6.5 ± 1.9)</td>
<td>428 (5.9 ± 1.3)</td>
</tr>
<tr>
<td>Fertilization rate</td>
<td>78%</td>
<td>54%</td>
<td>677%</td>
</tr>
<tr>
<td>Embryos per ET (mean ± SD )</td>
<td>3.0 ± 0.43</td>
<td>2.99 ± 0.65</td>
<td>3.02 ± 0.24</td>
</tr>
<tr>
<td>Implantation rate</td>
<td>22.52%</td>
<td>21.42%</td>
<td>20.53%</td>
</tr>
<tr>
<td>Clinical pregnancies/pregnancy rate</td>
<td>46 (54.8%)</td>
<td>51 (56.7%)</td>
<td>43 (52.4%)</td>
</tr>
</tbody>
</table>

(Mansour et al., 2008)

Table 2. Rate of acrosome reaction in relation to the sperm incubation time

<table>
<thead>
<tr>
<th>Sperm incubation time</th>
<th>Semen parameters (mean ± SD)</th>
<th>Total number of sperm heads studied</th>
<th>Number of sperm heads with acrosomal reaction</th>
<th>Rate of acrosomal reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hour</td>
<td>Group: 25.6% ± 1.15% total; Motility 11.2% ± 5.5%; Abnormal forms 79.5% ± 11.2%</td>
<td>106</td>
<td>79</td>
<td>21.6% ± 2%</td>
</tr>
<tr>
<td>3 hours</td>
<td>Group: 68.2% ± 4.8% total; Motility 12.5% ± 4.4%; Abnormal forms 78.6% ± 9.8%</td>
<td>398</td>
<td>122</td>
<td>40.9% ± 4%</td>
</tr>
<tr>
<td>5 hours</td>
<td>Group: 80.8% ± 5.5% total; Motility 12.3% ± 5.8%; Abnormal forms 78.6% ± 9.8%</td>
<td>251</td>
<td>173</td>
<td>68.2%</td>
</tr>
</tbody>
</table>

*Significant difference as compared to 3h group [OR = 0.63, 95% CI = 0.45 to 0.87, P = 0.005]. Significant difference as compared to 5h group [OR = 0.22, 95% CI = 0.15 to 0.31, P = 0.0001].

(Mansour et al., 2008)
Fig. 1: Transmission electron microscopic photomicrographs of 1-hour group. (A) Sperm head with disruption of the plasma membrane, intact acrosomal cap (AC) and a non-clear subacrosomal space. The chromatin is compact and dense within the nucleus (N) with no visible vacuoles (magnification X 31,760). (B) Acrosome-intact sperm head with the plasma membrane (PM) swollen away from the acrosome (X 26,467). (C) A sperm head with ruptured plasma membrane and intact non-reacted acrosome (AC) (magnification X 31,760) (Mansour et al., 2008).

Fig. 2: Transmission electron microscopic photomicrographs 3-hour group. (A) Two sperm heads, the upper head has a ruptured plasma membrane, intact non-reacted acrosome (AC), and a compact dense nucleus (N). The lower head shows complete disruption of the plasma membrane, and signs of early acrosomal reaction manifesting by the slight detachment of the acrosome (AC) from the nucleus and a clear subacrosomal space (SS). The nucleus (N) is compact with clear vacuoles (V) (magnification X 31,760). (B) Sperm heads with early some areas reacted acrosomes. Plasma membranes (PM) are swollen away from the nucleus and completely ruptured in some areas. The acrosome (AC) is slightly detached from the nucleus, with an irregular outer acrosomal membrane (OM) (magnification X 15,880) (Mansour et al., 2008).

Fig. 3: Transmission electron microscopic photomicrographs of 5-hour group. (A) (magnification X 26,467). (B) (magnification X 21,173) some of the sperm heads have reacted acrosomes, as manifesting by the complete disappearance or swelling of the plasma membranes (PM), detachment of the acrosome (AC) from the nucleus and internal vesiculation (V) adhering to the internal acrosomal membranes (IM).
The electron microscopic results in our study showed that the rate of acrosome reaction was time dependent, with a maximum of 5 hours. However, the fertilization rate was the highest when the spermatozoa were incubated for 3 hours.

Although the acrosome reaction increases with time there may be an increase in chromatin decondensation in the sperm head that adversely affects fertilization.

Therefore, based on the results of this study it is recommended to allow a 3 hour incubation period for spermatozoa before ICSI to obtain the best fertilization rate.
The oocyte "the beauty"

It is estimated that failed fertilization occurs in 2% – 3% of ICSI cycles.

Ebner T et al., 2004, Mahutte NG et al., 2003, Heindryckx B et al., 2005

In ICSI, because spermatozoa are injected inside the ooplasm, failure of pronuclear formation and division is most probably the result of failure of oocyte activation.

Many investigators tried different techniques for oocyte activation after ICSI to overcome this problem such as:
- Ionophore treatment
- Electrical oocyte activation

Rybochkin et al., 1997, Yanagida et al., 1999
Ionophore treatment for oocyte activation after ICSI resulted in the birth of a healthy baby in a case of previously failed fertilization due to globozoospermia

Rybochkin AV et al., 1997

Intracytoplasmic sperm injection followed by electrical oocyte activation resulted in the delivery of healthy twins for a couple with previously failed fertilization after ICSI.

Iwagida K et al., 1999

**Aim**

To estimate the value of the electrical activation of oocytes in patients with previously failed or limited fertilization after ICSI, as well as in patients with a possibility of failed fertilization as a results of teratozoospermia.

Mansour et al., 2009
Design

First: a pilot study on 10 patients who had previous total failure of fertilization in 11 ICSI cycles.

Second: a randomized controlled study on 241 infertile couples.

Sibling oocytes from each patient were randomly divided into two groups:
   1- Electroactivated group
   2- control group

Electrical activation was performed 30 minutes after ICSI. The time of inducing electrical oocyte activation was recommended to be as soon as possible because chromosome fragmentation was observed in 51% of unfertilized oocytes after ICSI.

Mansour, et al., 2009

Yanagida K. et al., 2004
The oocytes were suspended in 0.3M glucose drops, with pH at 7.3, and placed between 2 parallel electrodes (2 mm apart) in an electric slide chamber (BTX micro slide P/N 450, 0.5mm gap; BTX, San Diego, CA).

A double square direct-current pulse (130V, 50µc apart) was generated by using an electro cell manipulator (BTX) to achieve the desired field strength of 2.6 – 2.8 kv/cm.

The electrically stimulated oocytes immediately were transferred back to the tissue culture media to be rinsed then they were incubated under oil in 5% Co2 in air, at 37ºC.
### Table 1. Results of oocyte electroactivation in 10 ICSI cycles for patients with previous total failure of fertilization

<table>
<thead>
<tr>
<th>Case number</th>
<th>Oocytes Retrieved</th>
<th>MI II oocytes</th>
<th>2 PN oocytes</th>
<th>Embryos NO. transferred</th>
<th>No. of embryos Cryopreserved</th>
<th>Pregnancy results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>--</td>
<td>-ve</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>--</td>
<td>single</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>8</td>
<td>1</td>
<td>2</td>
<td>--</td>
<td>-ve</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>6</td>
<td>6</td>
<td>1</td>
<td>--</td>
<td>-ve</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>--</td>
<td>-ve</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>6</td>
<td>2</td>
<td>3</td>
<td>--</td>
<td>single</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>--</td>
<td>-ve</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>7</td>
<td>3</td>
<td>4</td>
<td>--</td>
<td>single</td>
</tr>
<tr>
<td>9</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>--</td>
<td>single</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>single</td>
</tr>
</tbody>
</table>

| Total       | 103              | 60            | 31           | 22                      | 6                            | 4 healthy babies |

Note: -ve = negative

Mansour, et al., 2009

### Table 2. The effect of electroactivation on the fertilization rates of sibling oocytes of 241 ICSI cycles with expected poor or failure of fertilization.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Oocytes</th>
<th>Electroactivated Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (mean ± SD) of metaphase II oocytes</td>
<td>1,435 (5.95 ± 2.9)</td>
<td>1,640 (6.8 ± 2.48)</td>
</tr>
<tr>
<td>No. (mean ± SD) of 2-pronuclear oocytes</td>
<td>1,116 (4.63 ± 2.3)</td>
<td>1,116 (4.63 ± 2.3)</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>No. (mean ± SD) of Degenerated oocytes</td>
<td>98 (1.73 ± 1.2)</td>
<td>98 (1.73 ± 1.2)</td>
</tr>
<tr>
<td>Degeneration rate (%)</td>
<td>5.9</td>
<td>5.9</td>
</tr>
</tbody>
</table>

a Odds ratio = 1.397, 95% confidence interval = 1.198 to 1.63, P < .001

b Odds ratio = 0.96, 95% confidence interval = 0.73 to 1.26, P = 0.821

Mansour, et al., 2009
Table 3. Pregnancy rates and outcome

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Embryos for ET derived from electro-activated group</th>
<th>Embryos for ET derived from control group</th>
<th>Embryos for ET derived from both groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of ET procedures</td>
<td>34</td>
<td>60</td>
<td>138</td>
</tr>
<tr>
<td>No. of clinical pregnancies</td>
<td>12 (44)</td>
<td>23 (48)</td>
<td>64 (46.4)</td>
</tr>
<tr>
<td>No. of miscarriages</td>
<td>3 (20)</td>
<td>3 (9)</td>
<td>4 (9.6)</td>
</tr>
<tr>
<td>Ectopic pregnancy</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of deliveries</td>
<td>12 (4 sets of twins, and 8 singletons, totaling 16 healthy babies, (9♂ + 7♀))</td>
<td>20 (6 sets of twins and 23 singletons, totaling 34 healthy babies, (16♂ + 18♀))</td>
<td>50 (1 set of triplets, 10 twins, and 47 singletons, totaling 70 healthy babies, (28♂ + 42♀))</td>
</tr>
</tbody>
</table>

The fertilization rate was significantly higher in the electroactivated group (68%) as compared with in the control (60%).

Most important, total failure of fertilization occurred in five cases in the control group; consequently, these patients would have lost their chance of embryo transfer and possibility of pregnancy if no electroactivation had been performed.
Since the commencement of this randomized controlled trial, we have been performing routine electroactivation for at least half of the oocytes in cases of severe OAT and azoospermia with 100% abnormal morphology or rare motile sperms, in which we expect poor or no fertilization.

Conclusions

1. The fertilization rates after ICSI depends on the oocyte and sperm quality, the ICSI technique itself, and the tissue culture conditions in the IVF lab.
2. Total failure of fertilization occurs in 2-3% of the ICSI cycles.

Conclusions

3. Preincubation of spermatozoa for 3h before ICSI achieved the highest fertilization.
4. Electro activation of oocytes after ICSI significantly improved fertilization in cases of previous failure of fertilization, teratospermia, and immotile spermatozoa.
• Heindryckx B, Van der Elst J, De Sutter P, Dhont M. Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. Hum Reprod 2005;20:2327-41.
• Heindryckx B, Van der Elst J, De Sutter P, Dhont M. Treatment option for sperm- or oocyte-related fertilization failure: assisted oocyte activation following diagnostic heterologous ICSI. Hum Reprod 2002;17:2642-9.
• Katayama M, Kasahara M, Miyake M. Fate of the acrosome in ooplasm in pigs after IVF and ICSI. Hum Reprod 2002;17:2635-44.
References (4)


The Egyptian IVF-ET Center

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  - S. Mostafa, Tech. Sc.
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  - A. El-Gendy, M. D.
  - E. Fathi, M. D.
  - I. Fahmy, M. D.
  - A. El-Gendy, M. D.
  - E. Fathi, M. D.
Longing for a girl: Gender selection by natural methods.

Annet M. Noorlander BSc, MSc
Gender Consult
Waalre
The Netherlands
European Society for Human Reproduction and Embryology
28th Annual Meeting Istanbul, Turkey 1 to 4 July 2012

Learning Objectives

• To learn why parents are interested in controlling the sex of their offspring.
• To learn about natural sex selection methods and their history.
• To learn about current research on natural sex selection methods.

Conflict of interest:

I am senior consultant at

Gender Consult
Consultancy bureau for natural sex selection
Waalre / The Netherlands
My personal story

• Always wanted a girl
• After 2 boys I decided to act
• I used my background in biology and nutrition
• Research into natural methods
• I applied diet and timing methods for myself
• Now I help others to accomplish their wish

Why do people have gender preferences?

• Traditional patrilineral inheritance:
  First-born must be a son / at least one son
• Economic burden from dowry:
  No daughter! (India, Bangladesh, Morocco)
• Family size restrictions: Boys! (China)
• Mother decides: Preference for a girl
• Family balancing: Western Europe, USA

Secundary Sex Ratio

Worldwide: 1.07 males per female
European Union: 1.06

• Azerbaijan 1.14 • Kazakhstan 0.94
• China 1.13 • Pacific Islands 1.02
• India 1.12 • African countries 1.03
• Vietnam 1.12
• Armenia 1.12
Sex ratio imbalance in China

Family Balancing: Fertility rate ratio for 1, 2, and 3 child families (Denmark)

Timing (Shettles) method

Y-bearing sperm is smaller, faster, more fragile
Intercourse close to ovulation favours boys
For a girl: Intercourse 2 – 3 days before ovulation
Supposed success rate: 75%

Very popular, widely applied for over 40 years
Problems in previous research:

Conflicting research results. Wilcox (1995):
“For practical purposes, the timing of sexual intercourse in relation to ovulation has no influence on the sex of the baby”

Unreliable estimation of time of ovulation

However: Modern urinary LH-based hometests determine ovulation very accurately

Diet method: History

- 1935 Herbst finds that sex ratio of *Bonellia viridis* is influenced by potassium.
- 1967-2007 Research on rats, sows, cattle: Sex ratio increases with Na, K intake, decreases with Ca, Mg.
- 1975-77 Retrospective diet surveys on mothers with at least 3 boys and on mothers with at least 3 girls confirms this.
PhD Thesis Michelle Duc, 1977

Retrospective analysis of diets:
1. Women with only ≥ 3 girls
2. Women with only ≥ 3 boys

Result:
Mothers of girls have higher Ca, Mg and lower Na, K intake than mothers of boys.

Stolkowski et al., Papa et al., Jeambrun, Devaure et al. (France, Canada, Portugal) investigated the effect of a preconceptional diet on baby sex
Multicenter experience: 500 women participated
75 – 85% were successful
Success depended largely on how strictly participants adhered to the diet
No objective quantification of compliance

Previous research diet method

<table>
<thead>
<tr>
<th>Researchers</th>
<th>Year</th>
<th>n</th>
<th>Successful</th>
<th>Success Rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stolkowski &amp; Lorrain</td>
<td>1980</td>
<td>260</td>
<td>212</td>
<td>82</td>
</tr>
<tr>
<td>Stolkowski &amp; Choukroun</td>
<td>1981</td>
<td>47</td>
<td>40</td>
<td>85</td>
</tr>
<tr>
<td>Papa et al.</td>
<td>1983</td>
<td>58</td>
<td>45</td>
<td>78</td>
</tr>
<tr>
<td>Jeambrun</td>
<td>1989</td>
<td>61</td>
<td>46</td>
<td>75</td>
</tr>
<tr>
<td>Devaure et al.</td>
<td>1989</td>
<td>72</td>
<td>58</td>
<td>81</td>
</tr>
<tr>
<td>Total</td>
<td>498</td>
<td>401</td>
<td>81</td>
<td></td>
</tr>
</tbody>
</table>
2000 Hudson and Buckley:

Sex ratio among 254 vegetarians is 0.815, compared to normal 1.06

2008 Mathews et al.:

You are what your mother eats

For a girl: low Na, K (n=720)

Results are debated

---

In my practice

- 85% wants a girl
- 15% wants a boy
- Family balancing is the main reason

---

Reasons for wanting a boy at GC

- Fathers find it important to have a son
- Male heir, family name
- Successor for firm or farm
- Having a son is important for Muslims
- Fathers can do man-things with a son
- Loss of a son earlier
Reasons for wanting a girl at GC

- Having a daughter is important for mothers
- Mother expects a closer relationship than with boy; identification with own gender
- Easier to handle, sweeter
- Mixed family, family balancing
- Loss of a daughter in pregnancy or as a baby

* A son is a son until he gets himself a wife, but a daughter is your daughter for the rest of your life. *
Break-down of GC clients

85% wants a girl
15% wants a boy

Previous boys
Previous girls

Number of previous boys
Number of previous girls

3% wants a second girl
3% has lost a girl
7% wants a second boy
5% has lost a boy

Treatment CG: Timing

• Training to predict ovulation 3-4 days in advance
• Monitoring 4-6 menstrual cycles:
  Basal body temperature, cervical mucus
  Cervical position, os diameter, texture, cycle length
• Gauge the observations with ovulation tests

Hormonal changes during cycle
Assessment of cervical mucus

Opaque, sticky

Fertile, stretchy

Clear, stretchy

After ovulation

Temperature Chart

Ovulation Tests

Negative

Positive
CG Treatment: Diet

- Mother follows diet ≥ 9 weeks prior to conception
- Low Na, high Ca using normal food products
- Supplemented with Ca, Mg, vitamin D
- At least 3 serum analyses for Na, K, Ca, Mg:
  - Before diet, after 5 weeks, after confirmed pregnancy
  - Diet stops after last blood sample

Sample food products

Scientific research GC

- Research started in 2001
- Cooperation with University of Maastricht (NL) and Delft University of Technology (NL)
- Couples with preference for a girl
- Publication in RBM Online 2010 (results 2001-6)
- This presentation is an update 2001-11
Study design

- A reference group of participants is used to construct a prediction rule, based on mineral blood serum values and timing data
- A validation group of participants is used to verify the validity of the prediction rule

Study population

- GC clients with a preference for a girl
- Period 2001 – 2011
- Healthy couples
- Age mother: 34.0 ± 3.3
- On average: 2.1 previous boys and 0.0 girls
Procedures / Basic requirements

- Diet must be followed uninterruptedly ≥ 9 weeks prior to conception
- Diet aims to increase Ca, decrease Na
- 3 blood samples: before starting diet, after 5 weeks, after confirmed pregnancy
- Proof of timing: Temperature charts/ovulation tests
- Proof of baby’s gender

Study overall

Discontinuations

<table>
<thead>
<tr>
<th>Reason for discontinuation</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miscarriage (19%)</td>
<td>18</td>
</tr>
<tr>
<td>Personal circumstances/divorce/not started</td>
<td>17</td>
</tr>
<tr>
<td>Impatience due to not becoming pregnant</td>
<td>12</td>
</tr>
<tr>
<td>Unplanned pregnancy before completing treatment</td>
<td>11</td>
</tr>
<tr>
<td>Illness</td>
<td>11</td>
</tr>
<tr>
<td>Lost to follow-up</td>
<td>9</td>
</tr>
<tr>
<td>Fertility problems</td>
<td>8</td>
</tr>
<tr>
<td>Second thoughts about having another baby</td>
<td>7</td>
</tr>
<tr>
<td>Finding the treatment too demanding</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
</tr>
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</table>
Partial data

<table>
<thead>
<tr>
<th>Reason</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>No post-pregnancy blood sample drawn</td>
<td>19</td>
</tr>
<tr>
<td>Diet was not started</td>
<td>11</td>
</tr>
<tr>
<td>Diet shorter than 9 weeks</td>
<td>10</td>
</tr>
<tr>
<td>No ovulation tests applied</td>
<td>6</td>
</tr>
<tr>
<td>Total (23 girls, 23 boys)</td>
<td>46</td>
</tr>
</tbody>
</table>

Prediction rule

Data from first 28 births was used to derive a prediction rule:

T1: Last intercourse $\geq 3$ days before ovulation

D1: $Na_2 + 20Ca_1 - 10Ca_2 \leq 163$ mM

D2: $Ca_2 \leq Ca_1 \Rightarrow Na_1 - Na_2 - 10Ca_1 + 10Ca_2 \geq 4$ mM

Validation of prediction rule

<table>
<thead>
<tr>
<th></th>
<th>Prediction rule satisfied</th>
<th>Prediction rule not satisfied</th>
<th>Total without prediction rule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference group</strong></td>
<td>91% 10♀, 1♂ n = 11</td>
<td>24% 4♀, 13♂ n = 17</td>
<td>50% 14♀, 14♂ n = 28</td>
</tr>
<tr>
<td><strong>Validation group</strong></td>
<td>79% 26♀, 7♂ n = 33</td>
<td>40% 19♀, 28♂ n = 47</td>
<td>56% 45♀, 36♂ n = 80</td>
</tr>
<tr>
<td><strong>Total research group</strong></td>
<td>82% 36♀, 8♂ n = 44</td>
<td>36% 23♀, 41♂ n = 64</td>
<td>55% 59♀, 49♂ n = 108</td>
</tr>
</tbody>
</table>
## Results

<table>
<thead>
<tr>
<th></th>
<th>Timing favouring girl</th>
<th>Timing favouring boy</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet correct</td>
<td>82%</td>
<td>50%</td>
<td>76%</td>
</tr>
<tr>
<td>n = 44</td>
<td>36♀, 8♂</td>
<td>5♀, 5♂</td>
<td>41♀, 13♂</td>
</tr>
<tr>
<td>Diet incorrect</td>
<td>36%</td>
<td>23%</td>
<td>33%</td>
</tr>
<tr>
<td>n = 41</td>
<td>15♀, 26♂</td>
<td>3♀, 10♂</td>
<td>18♀, 36♂</td>
</tr>
<tr>
<td>Total</td>
<td>60%</td>
<td>34%</td>
<td>55%</td>
</tr>
<tr>
<td>n = 85</td>
<td>51♀, 34♂</td>
<td>8♀, 15♂</td>
<td>59♀, 49♂</td>
</tr>
</tbody>
</table>

### Group satisfying the prediction rule

- The 44 participants that satisfied the prediction rule had ≥ 2 boys, no girls
- Success rate is 82% (P = 0.00001, as compared to usual 46%)
- Success rate is at least 70% (P = 0.05)

## Time to pregnancy

![Time to pregnancy graph]

- Number of pre-pregnancy menstrual cycles
- Number of pregnancies
The success rate for 3 days, 65%, is significant, p = 0.0015

Compliance with timing = 78%

Success rate of diet

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Girls</th>
<th>Success</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference group</td>
<td>11</td>
<td>10</td>
<td>91%</td>
</tr>
<tr>
<td>Validation group</td>
<td>46</td>
<td>33</td>
<td>72%</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>44</td>
<td>77%</td>
</tr>
</tbody>
</table>

Success rate of births satisfying diet prediction rule
Compliance of diet

• 74 out of 147 satisfy diet criterion: 50%

Effect of diet on serum values

<table>
<thead>
<tr>
<th></th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>141.6±2.6</td>
<td>4.40±0.48</td>
<td>2.38±0.11</td>
<td>0.89±0.13</td>
</tr>
<tr>
<td>After 5 weeks</td>
<td>139.9±2.1</td>
<td>4.40±0.41</td>
<td>2.41±0.09</td>
<td>0.91±0.13</td>
</tr>
<tr>
<td>End of diet</td>
<td>139.2±2.7</td>
<td>4.38±0.42</td>
<td>2.40±0.11</td>
<td>0.90±0.12</td>
</tr>
<tr>
<td>Reference range</td>
<td>135 – 150</td>
<td>3.6 – 5.4</td>
<td>2.1 – 2.7</td>
<td>0.70 – 1.10</td>
</tr>
</tbody>
</table>

Average serum values (n = 108)

Paired t-test:
- Decrease of Na (p = 0.0001)
- Increase of Ca (p = 0.05), Increase of Mg (p = 0.04)

IVF combined with diet

• All 3 babies from IVF preceded by diet were female

• In one case 10 out of 13 embryos were established to be female (77%, p =0.025)

• This preliminary IVF data suggests possible differential interaction of the oocyte with X- or Y-carrying sperm
New aspects of this research

- First study to investigate the efficacy of a combined diet and timing approach as a sex pre-selection technique
- Compliance with diet is quantified by mineral serum analyses
- Compliance with timing is quantified by ovulation tests

Conclusions

- Timing method increases the percentage of girls from 46% to about 60%
- Diet method increases the percentage of girls from 46% to about 75%
- Diet and timing method combined increase the percentage of girls from 46% to about 80%

Possible future research

- Continuation of this study for larger numbers
- Prediction rule for boys
- Effect of diet on IVF sex ratio?
Bibliography


Mark your calendar for the upcoming ESHRE Campus events

- Basic Semen Analysis Course in Greek Language  
  4-7 September 2012 - Athens, Greece

- Basic Genetics for ART practitioners  
  7 September 2012 - Rome, Italy

- Regulation of quality and safety in ART – the EU Tissues and Cells Directive perspective  
  14-15 September 2012 - Dublin, Ireland

- Basic Semen Analysis Course in Spanish language  
  18-21 September 2012 - Galdakano, Vizcaya

- GnRH-antagonists in ovarian stimulation  
  28 September 2012 - Hamburg, Germany

- The best sperm for the best oocyte  
  6-7 October 2012 - Athens, Greece

- Basic Semen Analysis Course in Italian language  
  8-11 October 2012 - Rome, Italy

- Accreditation of a preimplantation genetic diagnosis laboratory  
  11-12 October 2012 - Istanbul, Turkey

- Endoscopy in reproductive medicine  
  21-23 November 2012 - Leuven, Belgium

- Evidence based early pregnancy care  
  29-30 November 2012 - Amsterdam, The Netherlands

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(see “Calendar”)

Contact us at info@eshre.eu