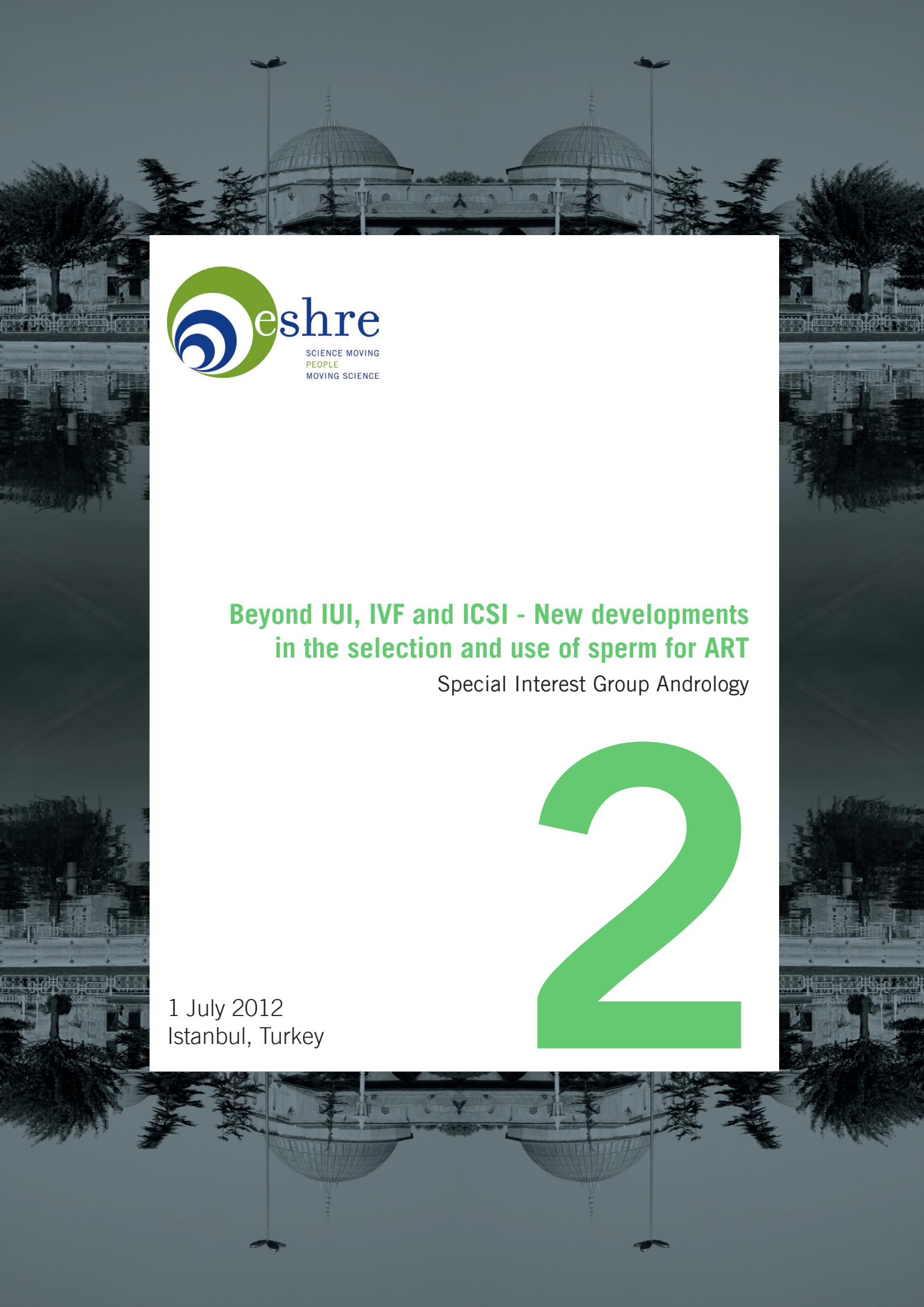

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Beyond IUI, IVF and ICSI - New developments in the selection and use of sperm for ART

Special Interest Group Andrology

2

1 July 2012
Istanbul, Turkey

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

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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

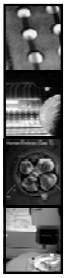
Sperm@work
Chairs: Herman Tournaye (Belgium) & Jose Castilla (Spain)

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

ESHRE SIG Andrology, Istanbul, Turkey, 7/01/2012

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.

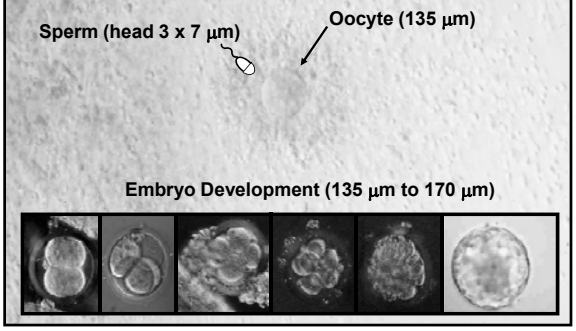
I was a major stockholder of Incept Biosystems, a start-up company working in the area of microfluidics and ART. Incept was purchased by Origio.

I am on the Scientific Advisory Board of Origio.

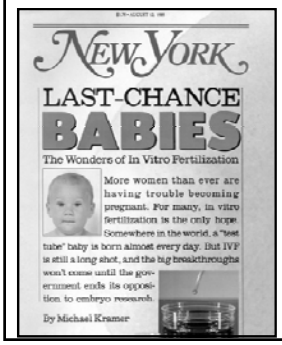
Learning Objectives / Outline

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



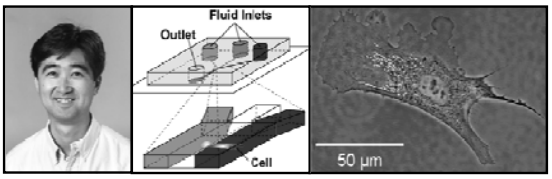
***In Vitro* Fertilization and Embryo Culture:**

- Media have changed substantially
- Processes have changed minimally (ICSI / extended culture)
- Hardware / related environments remain the same

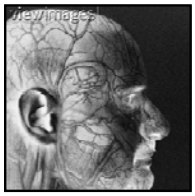
New Tools for Cell Based Treatment, Diagnosis, and Biology

October 2001 - “A Physics Revelation”

“Microfluidics, Laminar Flow, and Cell Biology”



Microfluidics



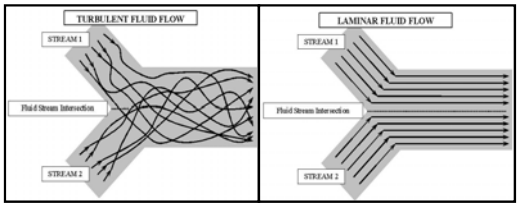
- study of physical principles of fluid behavior in a microenvironment and its application to chemistry, molecular biology, and cell biology

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

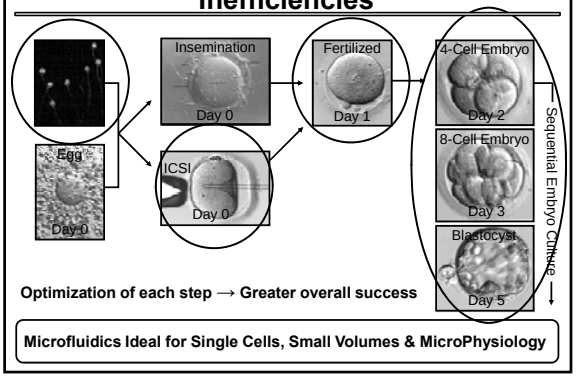
Turbulent Versus Laminar Flow

Fluid at the microscale exhibits laminar flow

Laminar flow is streamline and predictable



IVF: A Multi-Step Process With Stepwise Inefficiencies



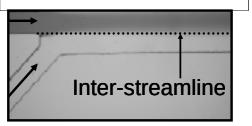
Optimization of each step → Greater overall success

Microfluidics Ideal for Single Cells, Small Volumes & MicroPhysiology

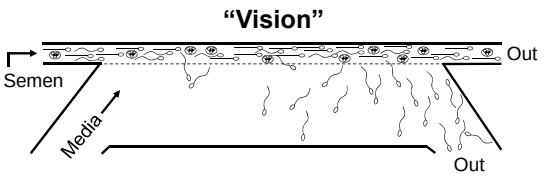
Could Microfluidics Be Useful In Isolation of Motile Sperm?

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

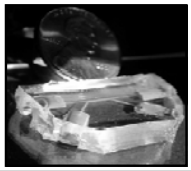


Microscale Integrated Sperm Sorter

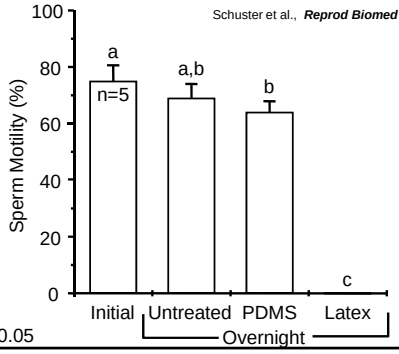


What was needed?

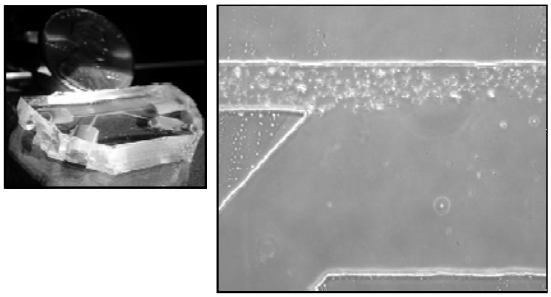
Sperm injection port
Fluid reservoir
Sperm collection port
Pump and/or power source



Polydimethylsiloxan and Sperm Survival



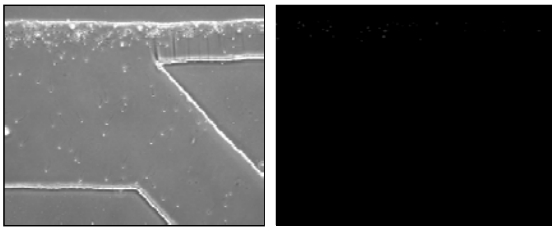
Microfluidic Sperm Separation



Cho et al., *Anal Chem;* **2003**
Schuster et al., *Reprod Biomed Online;* **2003**

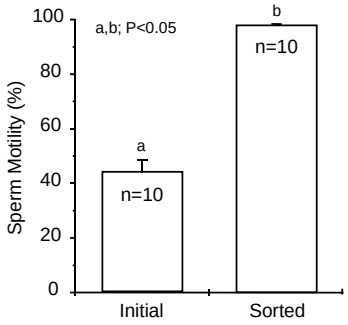
Microfluidic Motile Sperm Isolation

Live Sperm Go Right Dead Sperm Go Straight



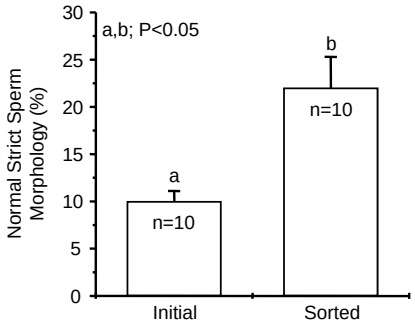
Cho et al., *Anal Chem;* **2003**
Schuster et al., *Reprod Biomed Online;* **2003**

Microfluidic Human Sperm Sorting



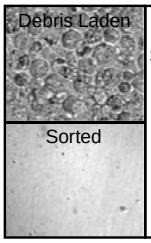
Schuster et al., ***Reprod Biomed Online;*** **2003**

Microfluidic Human Sperm Sorting



Schuster et al., ***Reprod Biomed Online;*** **2003**

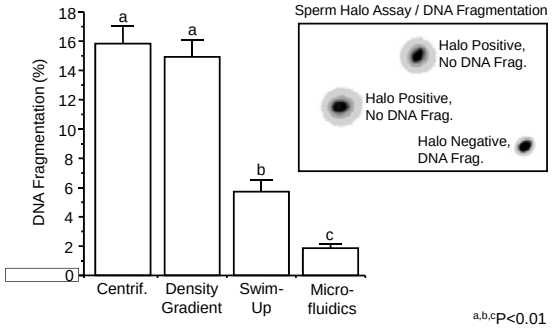
Isolation of Motile Spermatozoa from Debris Laden Samples



Sample	Motile Sperm / Round Cells: Inlet	Motile Sperm / Round Cells: Outlet	Motility (%): Outlet
A	1:10	64:1	96
B	1:10	100:1	99
C	1:10	32:1	95
D	1:10	12:1	97
E	1:10	34:1	99
F	1:10	200:1	100
Mean	1:10	33:1	98

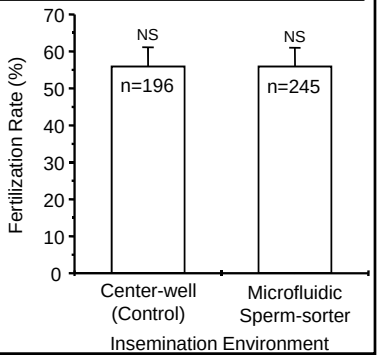
Schuster et al., ***Reprod Biomed Online;*** **2003**

Microfluidic Sperm Isolation Reduces DNA Fragmentation



$^{a,b,c}P<0.01$

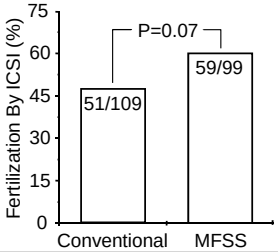
Can Microfluidic Sorted Sperm Fertilize?



Microfluidic Sperm Sorter (MFSS) : Clinical Trial Ongoing (Japan)

Nagoya University Hospital

- IRB approved / 40 couples
- inseminate 4 oocytes/cycle with MFSS isolated sperm



Microfluidic Sperm Sorter: Strengths and Weaknesses

Strengths:

1. Ease of use / disposable
2. No centrifugation needed
   - reduce physical or DNA damage
3. Toxicology testing
   - freestanding, no power source needed

Weaknesses:

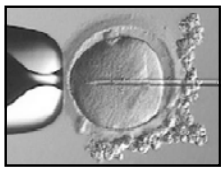
1. Only uses 40 μl semen
   - solution maybe multi-channels
2. Efficiency hard to predict
   - under some circumstances unimportant
3. Not compatible with current IVF insemination techniques

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

Intracytoplasmic Sperm Injection (ICSI)



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

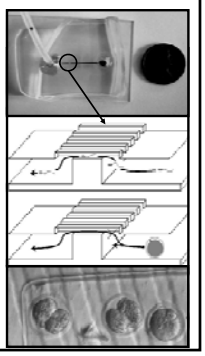
Micro-insemination Device

1. Removes randomness of sperm/egg interaction in conventional insemination

   - size constraint
   - direct delivery of sperm to egg
   - recirculation or re-insemination

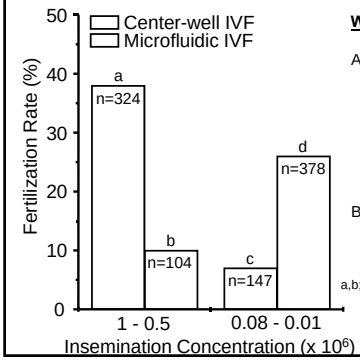
2. Unlike ICSI, it is noninvasive

3. Potential integration with sperm sorter



Suh et al., ***Human Reprod;*** **2006**

Micro-insemination



What is happening?

At high sperm concentration:

- high metabolic substrate usage
- concentrated degradative by-product

BAD for microfluidics

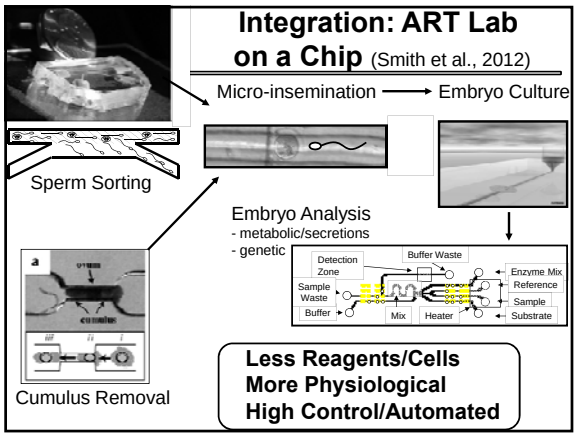
a,b; c,d $P<0.001$

Suh et al., ***Human Reprod;*** **2006**

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)



Less Reagents/Cells
More Physiological
High Control/Automated

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References

1. Cho B, Schuster T, Zhu X, Chang D, Smith GD, Takayama S. Passively driven integrated microfluidic system for separation of motile sperm. *Anal Chem* (2003) 75:1671-1675.

2. Schuster TG, Cho B, Keller LK, Takayama S, Smith GD. Isolation of motile sperm from semen samples using microfluidics. *Reprod BioMed* (2003) 6:1-10.

3. Suh RS, Phadke N, Ohl DA, Takayama S, Smith GD. In vitro fertilization within microchannels requires lower total numbers and lower concentrations of spermatozoa. *Human Reprod* (2006) 21:477-483.

4. Smith GD, Takayama S, Swain JE. Rethinking in vitro embryo culture: new developments in culture platforms and potential to improve assisted reproductive technologies. *Biol Reprod* (2012) 86:1-10.

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

Selecting spermatozoa; potential roles for electrophoresis and pharmacological enhancement

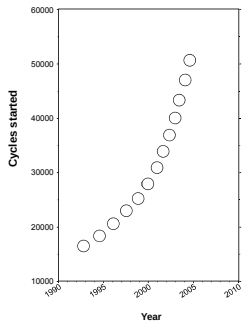
Learning objectives

As a result of this lecture attendees should appreciate that:

- **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 woman remaining childless at the end of their reproductive lives.**
- **One in 20 men are infertile**
- **One in every 30 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**

Clinical Problem

Male infertility is a major reason for ART

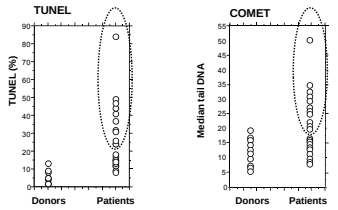
• **In a vast majority of infertile males sufficient numbers of spermatozoa are present to achieve fertilization: it is sperm function that is compromised**

• **Normal male reproductive function is not just about achieving fertilization, it is also about supporting normal embryonic development.**

FSA

Clinical Problem

DNA damage in the male germ line



Clinical Problem

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).

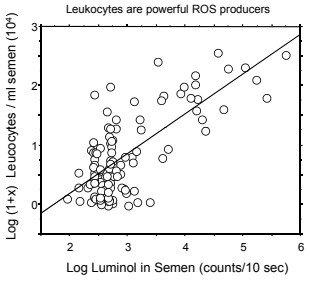
•**Reduced pregnancy rates following natural or assisted conception** (Loft, 2003, Duran 2002,; Bungum et al., 2004).

•**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** (Ji et al., 1997; Aitken and Krausz, 2001; Aitken, 2004).

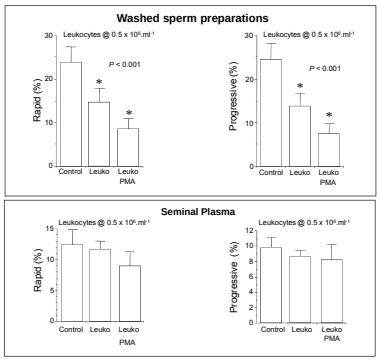
Select spermatozoa possessing low levels of DNA damage

The leukocyte problem



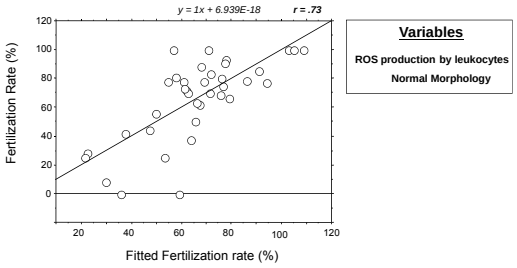
Aitken and Curry, 2011

Protection by Seminal Plasma



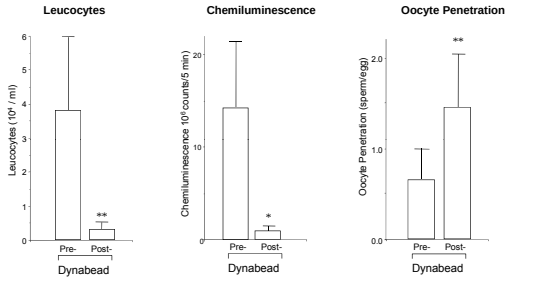
Aitken and Curry, 2011

Prediction of IVF Rates



Aitken and Curry, 2011

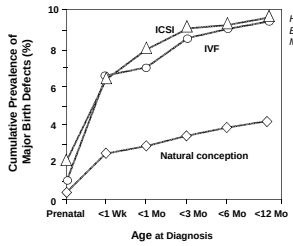
CD45^{+} Dynabeads and Leukocyte removal from Sperm prep^{ns}



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)



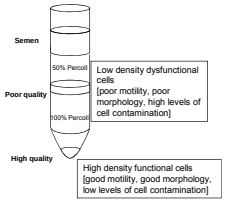
Hansen et al New England Journal of Medicine 2002

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**



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

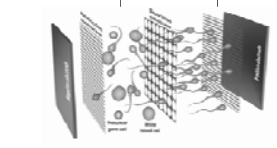
High density functional cells [good motility, good morphology, low levels of cell contamination]

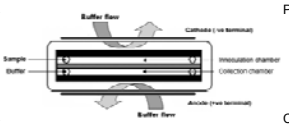
Density gradient centrifugation

Pros: Improved yields
Not dependent on sperm motility

Cons: Time consuming
Extraneous materials involved
Increase in DNA damage
Mechanical shearing forces

Spermsep CS10 System Sperm Cell Separation

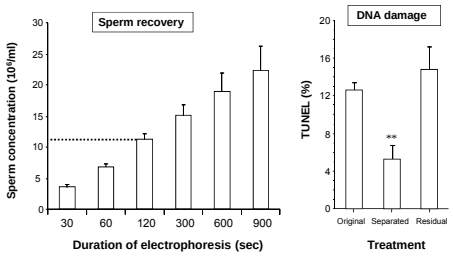




Pros: Simple
No contact with extraneous materials
Rapid
Effective with complex cellular mixtures
Good yield
Low levels of DNA damage

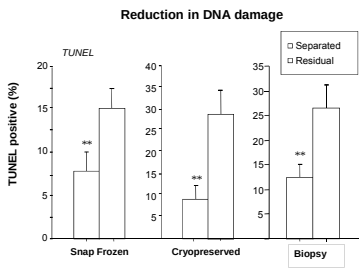
Cons: Limited field trial

Electrophoretic sperm isolation



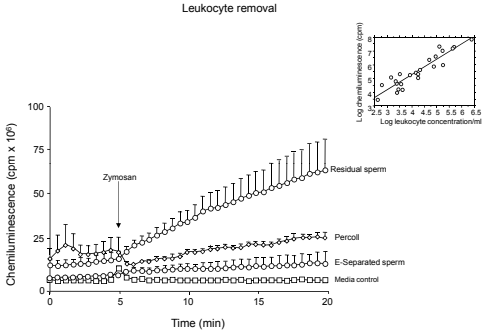
Ainsworth et al., 2005

Electrophoretic isolation of spermatozoa



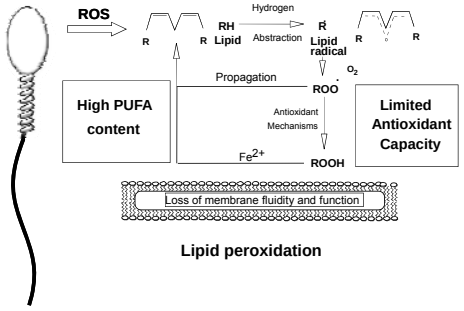
Ainsworth et al., 2005

Electrophoretic isolation of spermatozoa



Aitken and Curry, 2011

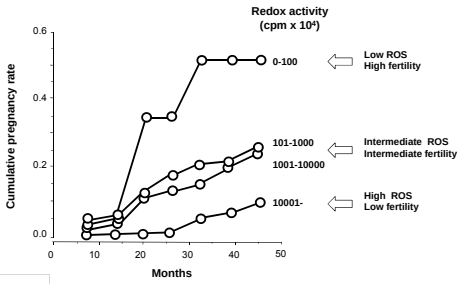
Susceptibility of spermatozoa to oxidative stress



Aitken and Curry, 2011

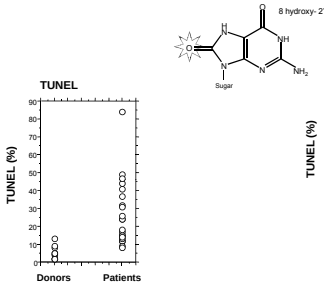
ROS and Spontaneous Pregnancy

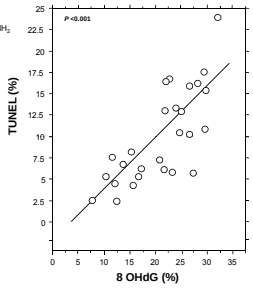
Spontaneous pregnancy in 139 couples characterized by normal female partner



Aitken and Curry, 2011

TUNEL and 8OHdG



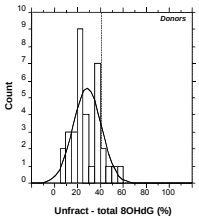


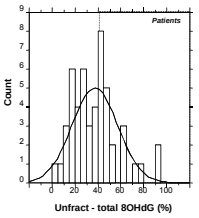
Aitken and Curry, 2011

Application of Youden's J to 8OHdG data

Donors **Patients**

14%





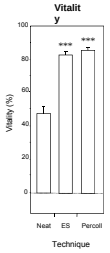
44%

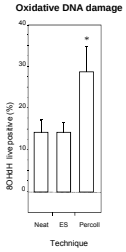
N.B These populations could not be separated on the basis of the conventional semen profile.

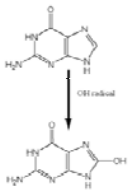
Aitken et al., 2010

Increased oxidative DNA damage with silicon based density gradient methods

CS10 vs Percoll







Aitken et al., 2011

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
  18% motility
  DNA damage - 26% TUNEL positive, 41% SCSA DFI
- **Post-separation:**

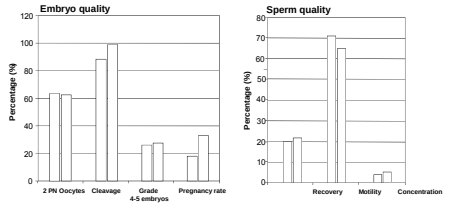
  62% vitality
  24% motility
  DNA damage - 14% TUNEL positive, 15% SCSA DFI
- Intracytoplasmic sperm injection (ICSI) was conducted using the electrophoretically isolated spermatozoa.
- Oocytes were fertilized and normal blastocysts were generated after 5 days of culture. Transfer of two embryos was 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

□Conventional methodology
□Electrophoretic sperm separation

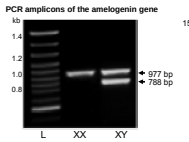


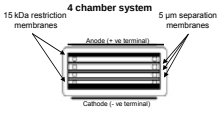
When all embryos are derived from one sperm preparation method

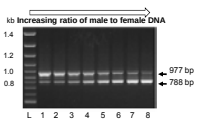
	Conventional	Electrophoretic
Embryo transfers	11	18
Pregnancies	2 (18%)	6 (33%)

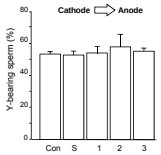
Fleming et al., 2008

Ratio of X and Y bearing spermatozoa



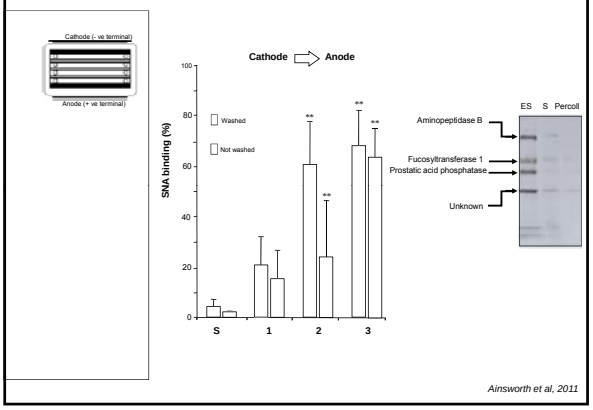






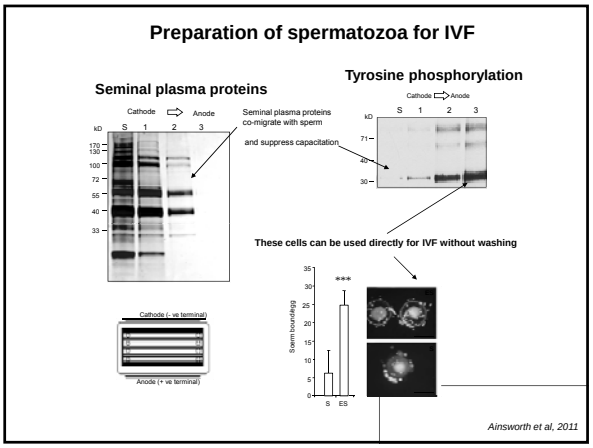
Ainsworth et al, 2011

Mechanism – sialation high quality sperm



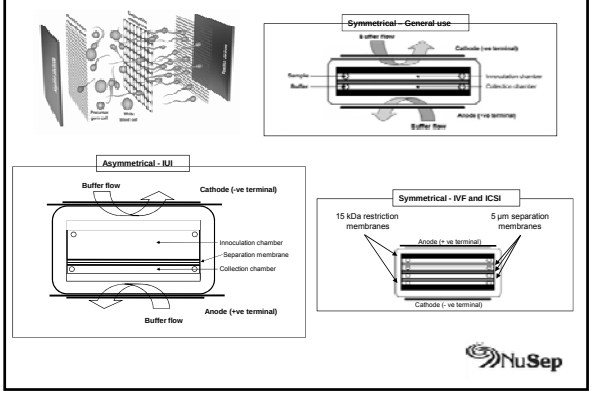
Ainsworth et al, 2011

Preparation of spermatozoa for IVF



Ainsworth et al, 2011

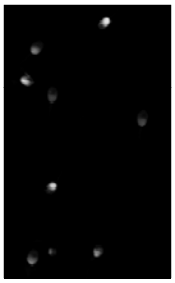
Electrophoretic Sperm Separation – cassette design



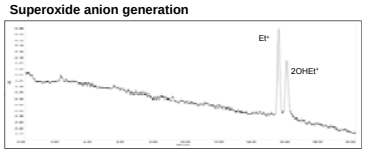
NuSep

Pharmacological impacts on sperm function

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

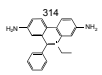


Superoxide anion generation



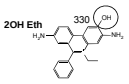
Peak 1

Eth



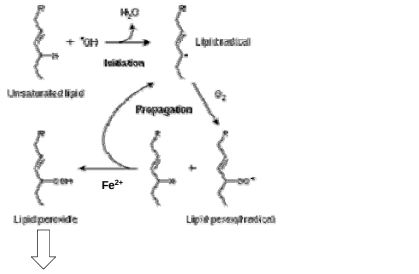
Peak 2

2OH Eth



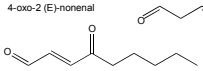
Superoxide specific

Lipid peroxidation generates powerful electrophiles



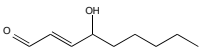
acrolein

4-oxo-2 (E)-nonenal

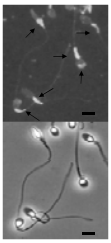


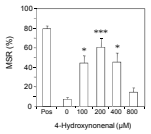
Malondialdehyde

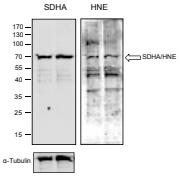
4-hydroxynonenal

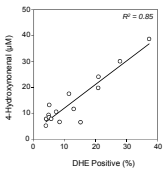


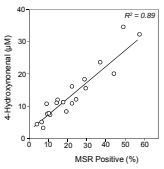
4HNE drives mitochondrial ROS generation











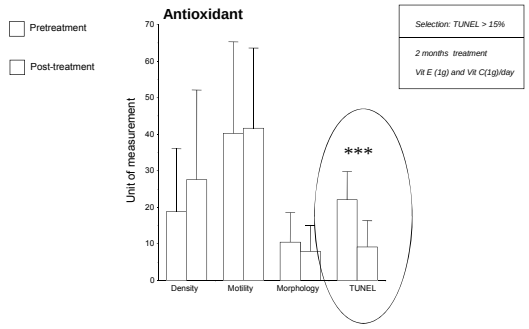
Hydrogen peroxide mediates DNA damage

$2H^+ + 2O_2^{\cdot-} = O_2 + H_2O_2$

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

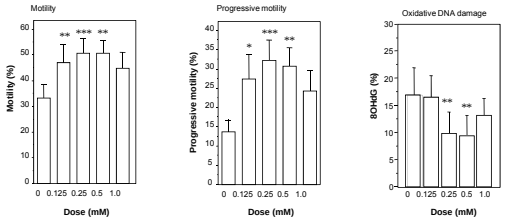
Impact of Vitamins E and C on DNA damage



Greco et al., 2005

Impact of penicillamine on sperm function

After 24 h incubation at 37° C



References -1

Ainsworth CJ, Nixon B, Aitken RJ. The electrophoretic separation of spermatozoa: an analysis of genotype, surface carbohydrate composition and potential for capacitation. Int J Androl. 2011 Oct;34(5 Pt 2):e422-34.

Ainsworth C, Nixon B, Jansen RP, Aitken RJ. First recorded pregnancy and normal birth after ICSI using electrophoretically isolated spermatozoa. Hum Reprod. 2007 Jan;22(1):197-200.

Ainsworth C, Nixon B, Aitken RJ. Development of a novel electrophoretic system for the isolation of human spermatozoa. Hum Reprod. 2005 Aug;20(8):2261-70.

Ainsworth CJ, Nixon B, Aitken RJ. The electrophoretic separation of spermatozoa: an analysis of genotype, surface carbohydrate composition and potential for capacitation. Int J Androl. 2011 Oct;34(5 Pt 2):e422-34.
Aitken RJ. Founders' Lecture. Human spermatozoa: fruits of creation, seeds of doubt. Reprod Fertil Dev. 2004;16(7):655-6
Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. Reproduction. 2001 Oct;122(4):497-506.
Aitken RJ, Curry BJ. Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. Antioxid Redox Signal. 2011 Feb 1;14(3):367-81.

Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. Hum Reprod. 2010 Oct;25(10):2415-26.

Aitken RJ, Hanson AR, Kuczera L. Electrophoretic sperm isolation: optimization of electrophoresis conditions and impact on oxidative stress. Hum Reprod. 2011 Aug;26(8):1955-64.

Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, Guérin JF.Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum Reprod. 2003 May;18(5):1023-8.

References - 2

Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. Hum Reprod. 2004 Jun;19(6):1401-8.

Carrell DT, Liu L, Peterson CM, Jones KP, Hatasaka HH, Erickson L, Campbell B. Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. Arch Androl. 2003 Jan-Feb;49(1):49-55.

Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Hum Reprod. 2002 Dec;17(12):3122-8.

Fleming SD, Ilad RS, Griffin AM, Wu Y, Ong KJ, Smith HC, Aitken RJ Prospective controlled trial of an electrophoretic method of sperm preparation for assisted reproduction: comparison with density gradient centrifugation. Hum Reprod. 2008 Dec;23(12):2646-51.

Greco E, Romano S, Iacobelli M, Ferrero S, Baroni E, Minasi MG, Ubaldi F, Rienzi L, Tesarik J. ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. Hum Reprod. 2005 Sep;20(9):2590-4.

Hansen M, Bower C, Milne E, de Klerk N, Kurinczuk JJ. Assisted reproductive technologies and the risk of birth defects--a systematic review. Hum Reprod. 2005 Feb;20(2):328-38.

Ji BT, Shu XO, Linet MS, Zheng W, Wacholder S, Gao YT, Ying DM, Jin F. Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. J Natl Cancer Inst. 1997 Feb 5;89(3):238-44.

Loft S, Kold-Jensen T, Hjollund NH, Giwercman A, Gyllemborg J, Ernst E, Olsen J, Scheike T, Poulsen HE, Bonde JP. Oxidative DNA damage in human sperm influences time to pregnancy. Hum Reprod. 2003 Jun;18(6):1265-72.

Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum Reprod. 2002 Apr;17(4):990-8.

References - 3

Sakkas D, Urner F, Bizzaro D, Manicardi G, Bianchi PG, Shoukir Y, Campana A.Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. Hum Reprod. 1998 Dec;13 Suppl 4:11-9.

Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR, Thomas AJ. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. Fertil Steril. 2003 Jun;79 Suppl 3:1597-605.

Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. Fertil Steril. 2004 May;81(5):1289-95.

Wisborg K, Ingerslev HJ, Henriksen TB. IVF and stillbirth: a prospective follow-up study. Hum Reprod. 2010 May;25(5):1312-6.

Zini A, Sigman M. Are tests of sperm DNA damage clinically useful? Pros and cons. J Androl. 2009 May-Jun;30(3):219-29.

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CLINICA VALLE GIULIA, Rome
SALUS, Marostica
UMERTIDE, Perugia

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

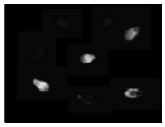
Early events

Late events

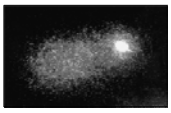
Failed fertilization
Abnormal zygote morphology
Impaired cleavage

Repeated implantation failures
Increased pregnancy loss

Sperm DNA integrity

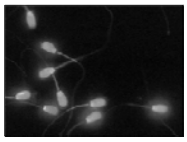


TUNEL

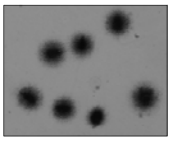


COMET

THESE METHODS CAN NOT BE CONDUCED IN REAL TIME



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 indipendent of basic sperm parameters.

Human Reproduction vol.10 no.5 pp.1123-1125, 1995

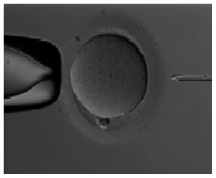
The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters.

Human Reproduction vol.11 no.5 pp.1019-1022, 1996

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

Peter Svalander[1], Ann-Helene Jakobsson, Ann-Sofie Forsberg, Anna-Carin Bengtsson and Matts 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

FERTILITY AND STERILITY
VOL. 79, N°1, JANUARY 2003

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

De Vos A, Van De VeldeH, JorisH, VerheyenG, DevroeyP, Van Steirteghem A.

Centre for Reproductive Medicine, University Hospital, Dutch-speakingBrussels Free University (VrijeUniversiteitBrussel), Belgium.

Sperm morphology and ICSI

Retrospective study 662 consecutive ICSI cycles

	Normal sperm morphology (ejaculated)	Abnormal sperm morphology (ejaculated)
No. Of oocytes injected	4,406	418
Fertilization rate (%)	72.5 ± 25.1	64.4 ± 38.0 *
Embryo quality	73.6 ± 29.8	72.5 ± 35.2
N°transfers	1226	41
Female age	34.1 ± 5.4	32.3 ± 6.7
Pregnancy rate (%)	37.0	22.0 *
Clinical pregnancy rate(%)	33.0	22.0 *
Implantation rate (%)	19.0 ± 31.7	11.2 ± 23.2 *
Live birth rate (%)	14.9 ± 28.4	7.9 ± 18.1 *

* Significantly different

De Vos *et al.*, 2003

REAL TIME FINE SPERM MORPHOLOGY ASSESSMENT

Intracytoplasmic Morphologically Selected Sperm Injection

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)

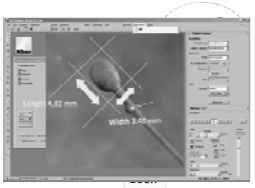
Introduction of a new concept to observe spermatozoa called 'motile-sperm organelle-morphology examination' (MSOME) and to evaluate the fine nuclear morphology of motile spermatozoa in real time.

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

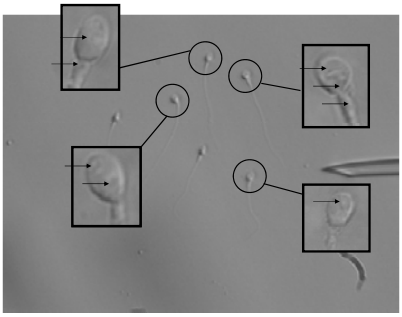
(no more than one vacuole / less than 4% of the nuclear area)

The average lenght and width limits in 100 spermatozoa with a normally looking nucleus, are estimated as follow:

- LENGHT: 4.75 ± 0.28 µm
- WIDTH: 3.28 ± 0.20 µm

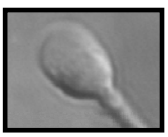
Bartoov et al., 2003

IMSI: Sperm assessment



IMSI: Sperm assessment

IMSI: Sperm assessment



Time expensive technique

Highly trained embryologists required

Additional cost to upgrade the equipment

IMSI: Clinical results

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?

Meta-analysis


Reproductive BioMedicine Online (2010) 21, 455–475

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REVIEW

Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: a meta-analysis

Amanda Souza Setti [a], Renata Cristina Ferreira [b], Daniela Paes de Almeida Ferreira Braga [a,b], Rita de Cássia Sávio Figueira [a,b], Assumpto Iaconelli Jr [b], Edson Borges Jr [a,b,*]

Studies included in the meta-analysis

Trial	Design	Participants	Numbers: Experimental (IMSI)	Numbers: Control (ICSI)	Outcomes
Bartoov et al. (2003)	Comparative	50 couples undergoing IMSI (male factor infertility, female age <37 years, more than three retrieved metaphase II oocyte in the last ICSI cycle, at least two previous consecutive failed ICSI cycles), matched with 50 couples undergoing ICSI	50	50	Fertilization rate, top-quality embryo rate, implantation rate, pregnancy rate, miscarriage rate
Berkovitz et al. (2006)	Comparative	80 couples (male factor infertility, female age <37 years, at least two previous consecutive failed ICSI cycles), matched with 80 couples undergoing ICSI	80	80	Fertilization rate, top-quality embryo rate, implantation rate, pregnancy rate, miscarriage rate
Antinori et al. (2008)	Randomized	446 couples (at least two previous diagnosis of severe oligoasthenozoospermia, at least 3 years of primary infertility, female age <35 years and undetected female factor) randomly allocated to receive ICSI and IMSI treatments	227	219	Fertilization rate, implantation rate, pregnancy rate, miscarriage rate

Souza Setti et al., 2010

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.

Souza Setti et al., 2010

IMSI: Prospective randomized study

168 Cycles

ICSI (87 cycles) **IMSI (81 cycles)**

Characteristics	ICSI	IMSI
Female age	28.80±4.08	29.67±4.03
Male age	32.53±4.87	33.97±5.52
Aetiology of infertility		
Male factor	39(48.1)	38(43.7)
Ovulatory	1 (1.2)	2 (2.3)
Tubal	10 (12.3)	7 (8.0)
Unexplained	24 (29.6)	30 (34.5)
Multiple factors	7 (8.6)	10 (11.5)

Balaban et al., 2011

Laboratory and clinical outcome

Outcome	ICSI	IMSI	P-value
Duration of ICSI procedure (min)	13.55 ± 5.43	20.54 ± 9.43	< 0.001
Fertilization rate (%)	80.97 ± 15.06	81.60 ± 10.65	NS
Grade 1 and 2 embryos on transfer day (%)	4.84 (63.95)	5.01 (66.44)	NS
Mean no.of embryos transferred	2.76 ± 0.46	2.72 ± 0.48	NS
Clinical pregnancy per initiated cycle (%)	36/81 (44.4)	47/87 (54.0)	NS
Live birth rate per initiated cycle (%)	31/81 (38.3)	38/87 (43.7)	NS
Implantation rate (%)	42/215 (19.5)	66/228 (28.9)	NS
Multiple pregnancy rate (%)	6/36 (16.7)	16/47(34.0)	<0.001

Balaban et al., 2011

Comparison of clinical pregnancy and implantation rates according to sperm characteristics

Live birth per initiated cycle (%)

	ICSI	IMSI	P value
No male factor	20/42 (47.6)	24/49 (49.0)	ns
Male factor	11/39 (28.2)	14/38(36.8)	ns
Sperm count			
< 1 million/ml	4/16 (25.0)	4/11(36.4)	ns
1-20 million/ml	7/22 (31.8)	10/27(37.0)	ns

Implantation rate (%)

	ICSI	IMSI	P value
No male factor	26/110 (23.6)	34/120 (28.3)	ns
Male factor	16/105 (15.2)	32/108 (29.6)	0.01
Sperm count			
< 1 million/ml	7/43 (16.3)	11/31 (35.5)	ns
1-20 million/ml	9/59 (15.3)	21/77 (27.3)	ns

Balaban et al., 2011

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?

	Experimental (n=28)	Control (n=28)
Retrieved oocytes	13.0 ± 5.0	12.1 ± 4.4
Injected oocytes	8.1 ± 3.6	8.4 ± 3.2
Fertilization rate (%)	68.7 ± 20.3	72.8 ± 18.5
Top quality embryos (%)	23.0 ± 31.1	27.1 ± 29.4
Embryos transferred	3.0 ± 1.3	3.2 ± 0.7
Pregnancy rates	18%	50% *
Abortion rate per pregnancies obtained	80%	7%*

Berkovitz et al. 2006

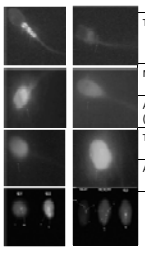
Does the presence of nuclear vacuoles influence the embryo's competence to develop to the blastocyst stage?

Characteristics	*Value*
No. of patients	25
Women's age (years, mean ± SD)	36.2 ± 2.5
No. of oocytes (mean ± SD)	247 (9.9 ± 1.6)
No. of MII oocytes (mean ± SD)	198 (7.9 ± 1.8)
No. of MII oocytes for injection (mean ± SD)	164 (6.6 ± 1.4)

Results *Type of injected spermatozoa*	*Grade I/II*	*Grade III/IV*	*P-value*
No. of injected oocytes (mean ± SD)	86 (3.4 ± 0.9)	78 (3.12 ± 1.0)	NS
Percentages (no.) of embryos per injected oocyte			
Zygotes	89.5 (77)	84.6 (66)	NS
Day-3 embryos	88.4 (76)	82.1 (64)	NS
Good quality day-3 embryos	43.0 (37)	30.8 (24)	NS
Blastocysts	60.5 (52)	3.8 (3)	<0.001
Good quality blastocysts	37.2 (32)	1.3 (1)	<0.001

Vanderzwalmen et al., 2008

Sperm morphology and IMSI and physiologic quality



Test	Whole sperm samples			TD patients, single sperm	
	Controls (n=10)	PO (n=10)	TD (n=10)	Group A (100 cells)	Group B (100 cells)
Mitosensor (%)	15.5 ± 6.1	31.6± 14.1[a]	48.7±15.3[bc]	13.3 ± 4.9	52.2 ± 14.7[e]
Acridine orange (%)	15.7 ± 6.1	29.8 ± 8.8[c]	77.9± 3.3[cd]	5.3 ± 3 .0	71.9 ± 11.1[e]
TUNEL (%)	14.0 ± 6.4	28.9± 12.7[a]	58.0± 1.1[b,c]	9.3 ± 4.8	40.1 ± 11.6[e]
Aneuploidies (%)	1.2 ± 0.4	1.3 ± 0.5	14.5± 8.4[c,d]	0.0	5.1 ± 3.1

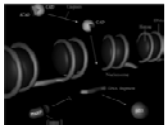
TD=testicular damage: P0= partialo bstruction
a=*P*< 0.01 versus controls; b=*P*< 0.01 versus PO; c=*P*< 0.001 versus controls;

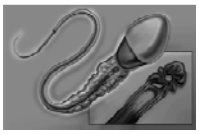
D=*P*< 0.001 versus PO; e=*P*< 0.001 versus group A.

Garolla et al., 2008

Nuclear vacuoles and sperm competence

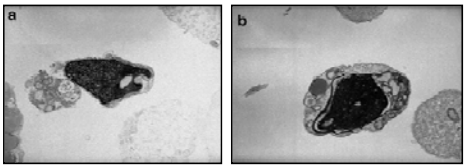
- DNA Integrity
- Mitochondrial function
- Chromosomal aberrations





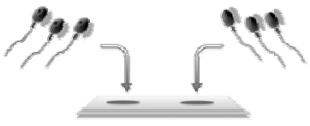
What Are Sperm Vacuoles?

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



Palermo et al., 2011

Are sperm vacuoles responsible for DNA damage?



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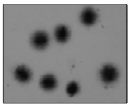
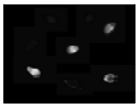
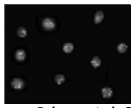


SCD



TUNEL



Palermo et al., 2011

Sperm DNA fragmentation

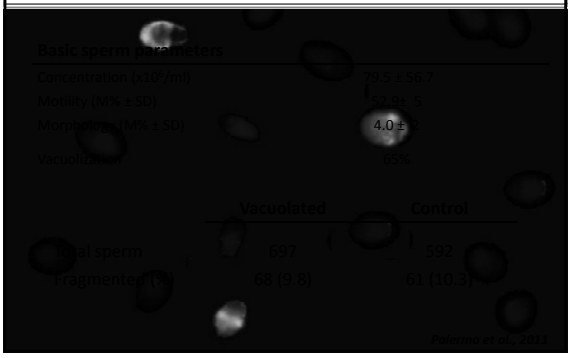
Basic sperm parameters

Concentration (x10^6/ml)	63.5 ± 26.3
Motility (M% ± SD)	56.9± 1.7
Morphology (M% ± SD)	3.4 ± 3.2
Vacuolization	67%

	Vacuolated	Control
Total sperm	576	486
Fragmented (%)	23 (3.9)	22 (4.5)

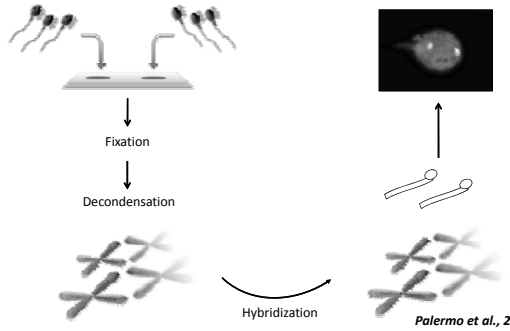
Palermo et al., 2011

Sperm DNA fragmentation



Palermo et al., 2011

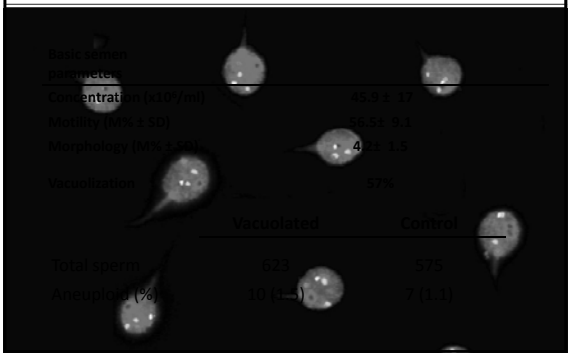
Fluorescent *in situ* hybridization



Palermo et al., 2011

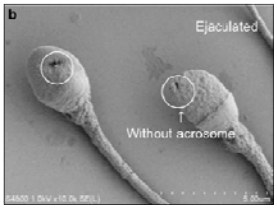
Chromosomal content

(X, Y, 13, 15, 16, 17, 18, 21, 22)

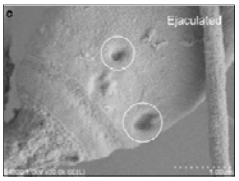


Basic semen parameters	Vacuolated	Control
Concentration (x10⁶/ml)	45.9 ± 17	
Motility (M% ± SD)	56.51 ± 9.1	
Morphology (M% ± SD)	4.2 ± 1.5	
Vacuolization	57%	
Total sperm	623	575
Aneuploid (%)	10 (1.6)	7 (1.1)

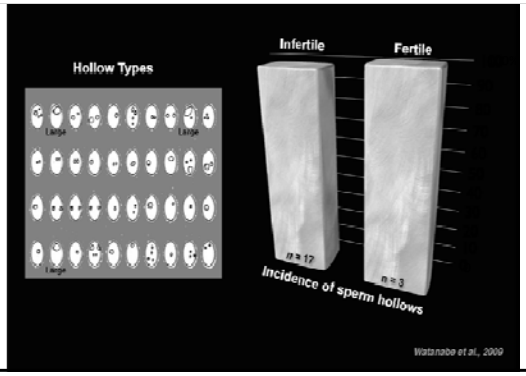
Are we really speaking about vacuoles?



Surface "vacuole" or hollows on the surface



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



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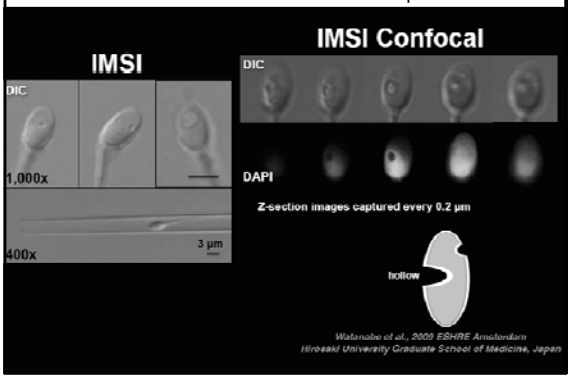

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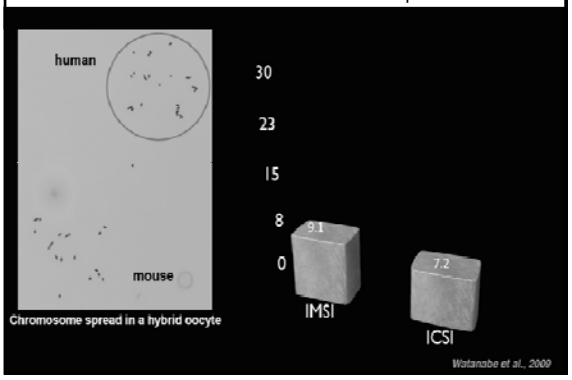

Corrective role of the oocyte

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

-**Nagy et al., 1995**
-**Svalander et al., 1996**
-**Bartoov et al. 2001, 2002, 2003**
-**De Vos et al., 2003**
-**Virro, et al. 2004**
-**Berkovitz 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**

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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

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

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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

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Sperm selection: why?

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

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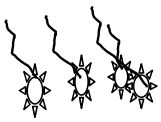
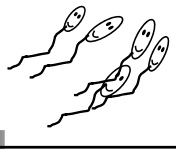
Sperm selection for ART: how?



"Negative" selection (damaged sperm bind to membrane receptors; e.g. Annexine-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



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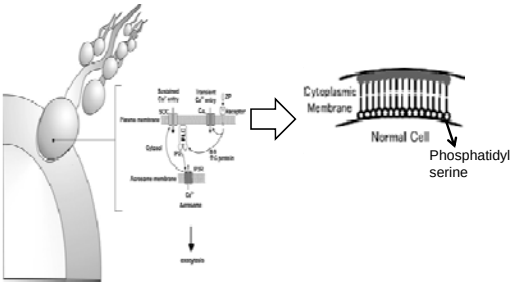
Sperm membranes are highly polyunsaturated; they have a specific constitution and function: changes are necessary for acrosom reaction, ZP recognition and oocyte for fusion



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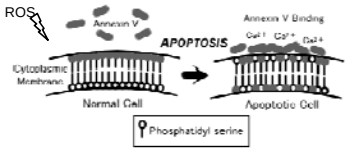
"Negative" markers and sperm selection:

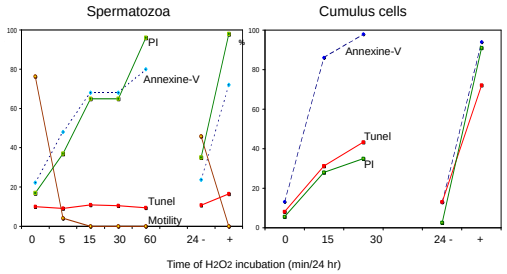
principles and theory of the Annexine V binding test

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



UMC St Radboud ESHRE 2000

Damage induced by ROS



UMC St. Radboud, Fertility laboratory - ESHRE 2005

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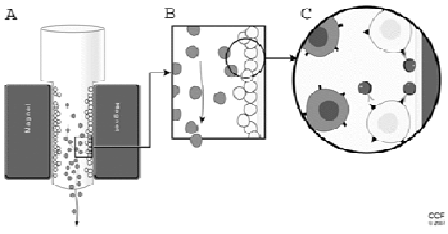
Magnetic microbeads are conjugated with Annexine V: damaged sperm binds to Annexine V and are retained in a magnetic field



Utility of Magnetic Cell Separation as a Molecular Sperm Preparation Technique
TAMER M. Said, et al. Journal of Andrology, Vol. 29, No. 2, March/April 2008

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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 pentration and hamster ICSI-test

MACS-Annexine V negative sperm:

- higher % motile sperm
- lower % sperm apoptotic makers
- higher % oocyte penetration
- no difference in chromatine decondensation after ICSI

Said et al. Reprod. Biomed Online 2005 10(6):740-6
Said et al. Biol Reprod. 2006 74(3):530-7

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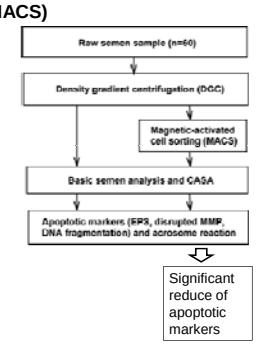
II. MACS –Annexine V for diagnostic testing (density gradient centrifugation and MACS)

Patients: 60 couples with unexplained infertility and failed IUI



Significant reduce of apoptotic markers

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

Lee et al. Hum. Reprod. 2010 25 (4): 839-46

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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

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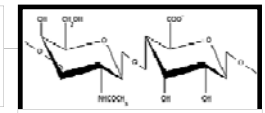
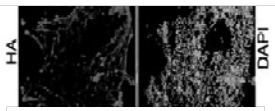
"Positive" markers and sperm selection: principles and theory of the Hyaluronic acid (HA) binding test

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



Hyaluronic acid is a polysaccharide of the glycosaminoglycans class.



Zhuo and Kimata (2001) Cell Structure & function

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

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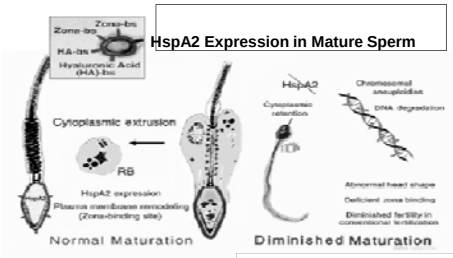
Correlation between HspA2 expression in mature sperm and HA binding capacity:

increased sperm maturity, viability and diminished aneuploidy rates

HspA2 Expression in Mature Sperm



Huszar et al. Biol.Reprod 2000
Huszar et al. Fert.Steril.2003
Kovanci et al. Hum Reprod. 2001

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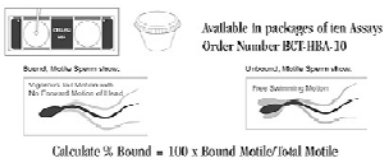
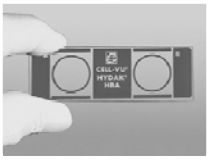
The HA- binding: the diagnostic test



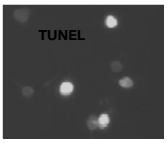
Evaluation of HA-binding and sperm integrity

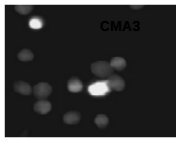
HBA-slides (provided by MidAtlantic Diagnostics)
25 Semen samples:
12 normospermia
13 abnormal (OAT or combinations)



%HA binding= **# motile bound sperm x 100**
 # total motile sperm

Tests

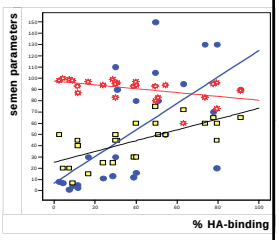




* original sample
* sperm fraction after gradient centrifugation
* HA-bound fraction

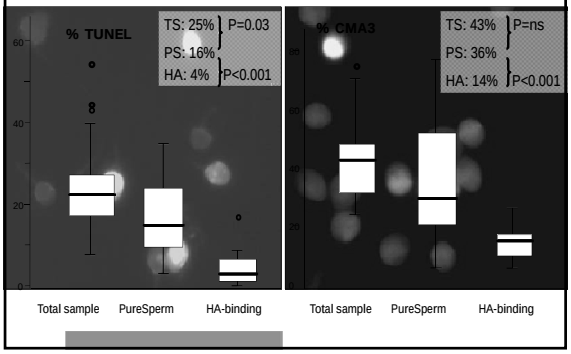
Results (1)
% HA-binding and semen parameters

Pearson correlations
- Concentration: r= 0.551 *P*=0.004
- % 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



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Zeta and HA-binding : evaluation of sperm integrity

Razavi et al. Andrologia 2010 42(1):13-9

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)

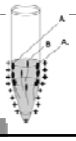

Diagram for sperm selection using **zeta potential**. Sperm suspension is pipetted into a positively charged tube. Negatively charged mature sperm (**A**) will adhere to the tube, while non-mature sperm (**B**) will not adhere and are discarded.

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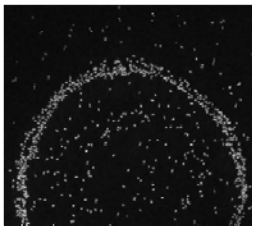
HA- bound sperm: DNA damage evaluated by acridine orange fluorescence (AOF)

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

Green fluorescence (double-strengs DNA)

- Total fraction 54%
- HA-bound sperm (in spot) : 99%



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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

Reprod Biomed Online. 2009 Feb;18(2):177-83.
Selectivity of hyaluronic acid binding for spermatozoa with normal Tygerberg strict morphology.
Prinosilova P, Kruger T, Sati L, Ozkavukcu S, Vigue L, Kovanci E, Huszar G.

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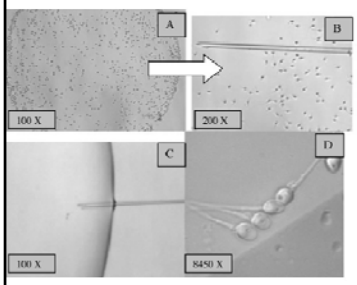
HA-bound sperm and morphology with MSOME evaluation



16.592 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

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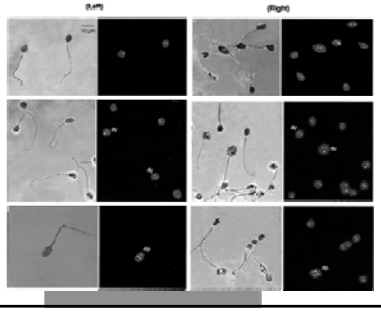
HA-bound sperm and aneuploidy

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



Jakab, et al. Fertil Steril 2005 84(6):1665-73

Huszar et al. Reprod Biomed Online 2007 14(5):650-63

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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

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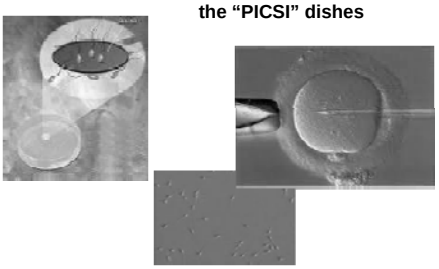
HA-binding and predictive value for ART:

the "PICSI" dishes



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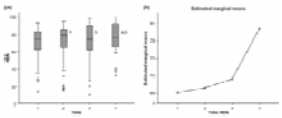
I. HA and ART outcome: prospective study

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

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

Kovacs et al. J. Assist Reprod Genet 2011 28(1): 49-54

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

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III. HA and ICSI outcome: prospective randomized study

Van den Bergh et al . Reprod. Biomed. Online 2009 19(6): 796-801
Nasr-Esfahani et al. J Assit Reprod Genet 2008 25 (5):197-203

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

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IV. HA and ART outcome: freezability of sperm

Yogev et al. Fertil Steril 2010 93(1): 154-8

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

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Va. HA and IVF outcome

Ye et al. Hum Reprod. 2006 21(6): 1545-50

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

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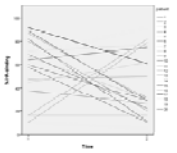
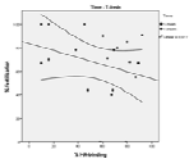
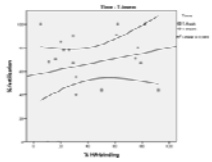
VI. HA and IVF outcome: retrospective study

(20 samples, Time1= 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





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VII. HA and IVF outcome (under Italian law)

Tarozzi et al. Reprod Biomed Online 2009 (19) suppl 3:35-43

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

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VIII. HA and ICSI outcome (under Italian law)

1) Tarozzi et al. Reprod Biomed Online 2009 (19) suppl 3:35-43

2) Parmegiani et al. Fertil Steril 2010 93(2):598-604

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

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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?

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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-bond 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

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References list

Said TM, Land JA. Effects of advanced selection methods on sperm quality and ART outcome: a systematic review. Hum Reprod Update. 2011 Nov-Dec;17(6):719-33.

Yagci A, et al. Spermatozoa bound to solid state hyaluronic acid show chromatin structure with high DNA chain integrity: an acridine orange fluorescence study. J Androl. 2010 Nov-Dec;31(6):566-72.

Prinosilova P et al. Selectivity of hyaluronic acid binding for spermatozoa with normal Tygerberg strict morphology. Reprod Biomed Online. 2009 Feb;18(2):177-83.

Said TM et al. Utility of magnetic cell separation as a molecular sperm preparation technique. J Androl. 2008 Mar-Apr;29(2):134-42.

Huszar G, et al. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects.Reprod Biomed Online. 2007 May;14(5):650-63.

Said TM et al. Selection of nonapoptotic spermatozoa as a new tool for enhancing assisted reproduction outcomes: an in vitro model.Biol Reprod. 2006 Mar;74(3):530-7.

Said TM et al. Advantage of combining magnetic cell separation with sperm preparation techniques. Reprod Biomed Online. 2005 Jun;10(6):740-6.

Lee TH et al. Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility. Hum Reprod. 2010 Apr;25(4):839-46.

Huszar G, et al. Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. Fertil Steril. 2003 Jun;79 Suppl 3:1616-24.

Naz RK , Leslie MH. Sperm surface protein profiles of fertile and infertile men: search for a diagnostic molecular marker Arch Androl. 1999 Nov-Dec;43(3):173-81.

Petersen CG, et al. Efficacy of hyaluronic acid binding assay in selecting motile spermatozoa with normal morphology at high magnification.Reprod Biol Endocrinol. 2010 Dec 3;8:149.

Nijs M, et al. Relationship between hyaluronic acid binding assay and outcome in ART: a pilot study. Andrologia. 2010 Oct;42(5):291-6.

Kovacs P, et al. The role of hyaluronic acid binding assay in choosing the fertilization method for patients undergoing IVF for unexplained infertility. J Assist Reprod Genet. 2011 Jan;28(1):49-54.

Razavi SH, et al. Evaluation of zeta and HA-binding methods for selection of spermatozoa with normal morphology, protamine content and DNA integrity. Andrologia. 2010 Feb;42(1):13-9.

Van Den Bergh MJ, et al. Pronuclear zygote score following intracytoplasmic injection of hyaluronan-bound spermatozoa: a prospective randomized study. Reprod Biomed Online. 2009 Dec;19(6):796-801.

Yogev L, et al. Assessing the predictive value of hyaluronan binding ability for the freezability potential of human sperm. Fertil Steril. 2010 Jan;93(1):154-8. Epub 2008 Nov 19.

Nasr-Esfahani MH, et al. Evaluation of sperm selection procedure based on hyaluronic acid binding ability on ICSI outcome. J Assist Reprod Genet. 2008 May;25(5):197-203

Ghosh I, et al. Hyaluronan binding protein-1: a modulator of sperm-oocyte interaction. Soc Reprod Fertil Suppl. 2007;63:539-43.

Ye H, et al. Relationship between human sperm-hyaluronan binding assay and fertilization rate in conventional in vitro fertilization. Hum Reprod. 2006 Jun;21(6):1545-50.

Jakab A, et al. Intracytoplasmic sperm injection: a novel selection method for sperm with normal frequency of chromosomal aneuploidies. Fertil Steril. 2005 Dec;84(6):1665-73.

Ranganathan S, et al. Evidence for presence of hyaluronan binding protein on spermatozoa and its possible involvement in sperm function. Mol Reprod Dev. 1994 May;38(1):69-76.

Tarozzi N, et al. Sperm-hyaluronan-binding assay: clinical value in conventional IVF under Italian law. Reprod Biomed Online. 2009;19 Suppl 3:35-43.

Parmegiani L, et al. Efficiency of hyaluronic acid (HA) sperm selection. J Assist Reprod Genet. 2010 Jan;27(1):13-6.

Parmegiani L, et al. "Physiologic ICSI": hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Fertil Steril. 2010 Feb;93(2):598-604.

May the force be with you

Using immotile sperm in ART

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Universitair Ziekenhuis Brussel

Vrije Universiteit Brussel

Centrum voor Reproductieve Geneeskunde

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

⇓

Natural conception

Migration vagina → Fallopian tubes

Penetration of cumulus complex

Binding to zona pellucida

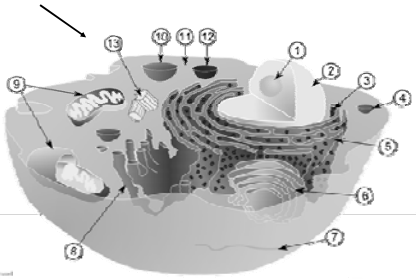
Introduction

Origin of motility

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

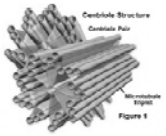
Sperm ultrastructure

Centrosome = microtubule organizing centre (MTOC)

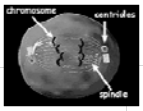


Sperm ultrastructure

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



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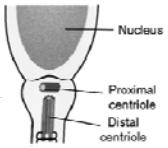
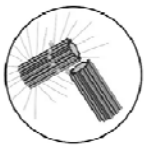
sperm flagellum

- Specialized structure

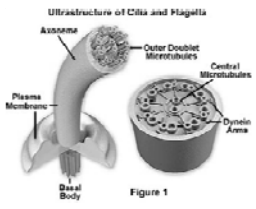
Sperm ultrastructure

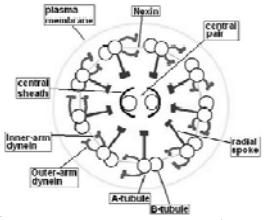
- **Role of the sperm centrosome**
  - Proximal centriole → sperm aster after fertilization
    → spindle formation (mitosis)
  - Distal centriole → tail during spermiogenesis





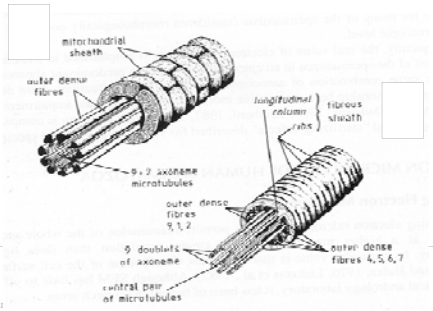
Sperm ultrastructure





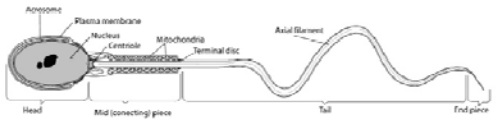
Axonemal structure

Sperm ultrastructure



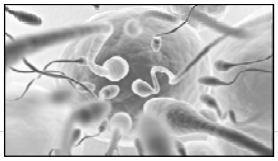
Energy metabolism

- **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**

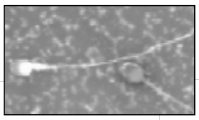


Diagnostic semen analysis

- **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

Diagnostic semen analysis

- **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 ***Eliasson et al. 1977***

Absolute asthenozoospermia

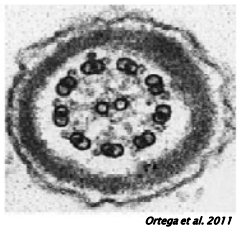
- **Aetiology?**
  - Ultrastructural defects
    - Congenital
    - Inherited
  - Necrozoospermia
    - Genital infection
    - Oxidative stress
    - Cryopreservation
    - ASA
    - Metabolic disorders
    - Exposure to environmental polluents
    - Prolonged period of anejaculation
  - Unexplained

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

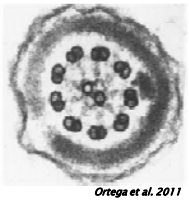


Ortega et al. 2011

Normal axoneme

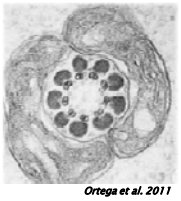
Ultrastructural defects

Transmission electron microscopy



Ortega et al. 2011

Absence of dynein arms



Ortega et al. 2011

Absence of central microtubuli (9+0)

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 ultrosonography, 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)*

Table II. Results of intracytoplasmic sperm injection in relation to sperm motility

	Total	MOT:1 ♂	MOT:2 ♂	MOT:3 >0 to ≤5	MOT:4 >5 to ≤50	MOT:5 >50	*P* value from Kruskal–Wallis
No. of cycles	901	12	54	19	479	337	
No. of injected oocytes	9117	176	501	252	4894	3412	
% of intact oocytes ±SD	89.0 ± 14.4	88.3 ± 10.9	92.3 ± 12.6	86.9 ± 13.6	89.3 ± 14.0	88.3 ± 15.3	NS
Pronuclear status							
% of intact oocytes ±SD							
0 PN	21.0 ± 22.2	74.6[a] ± 20.4	30.1[bd] ± 28.0	21.7[d] ± 18.1	20.0[cf] ± 21.6	12.5[af] ± 19.2	<0.0001
1 PN	3.9 ± 8.7	13.35 ± 13.6	6.1 ± 9.3	4.7 ± 8.9	3.8[e] ± 8.5	3.5[e] ± 6.9	<0.0001
2 PN	70.1 ± 23.4	10.9[b] 12.1	60.3[ab] ± 27.2	68.2[d] ± 22.3	70.2[cf] ± 22.3	73.8[cf] ± 20.9	<0.0001

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**

↓

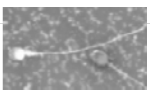
- **The lab must solve the problem**
- **Challenge = discriminate immotile live sperm from immotile dead sperm**

Absolute asthenozoospermia

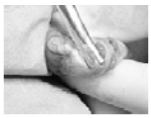
- **100% immotility in the ejaculate**
- **Two possibilities**
  - Viability = 0% ⇒ necrozoospermia
  - Viability > 0%
    - How to select viable sperm for ICSI?
    - Exposure to dyes

⇓

Unsuitable for ICSI



Necrozoospermia / low viability rate



- **Second semen sample**
- **Go to the testis**
  - Increased chance to find live/motile sperm
  - No garantuee
  - Overnight incubation ≠ valid option
    - May improve the quality of motility ***Emiliani et al. 2000***
    - 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:

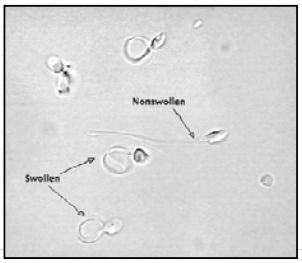
1. ***Hypo-osmotic swelling test (HOST)***
2. ***Exposure to motility enhancers (ME)***
3. ***Mechanical touch technique (MTT)***
4. ***Laser-assisted immotile sperm selection (LAISS)***
5. ***Birefringence polarisation microscopy (BPM)***

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:**

1. Hypo-osmotic swelling test



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
    26% with randomly-selected sperm
  - Embryo development: 39% with HOS-selected sperm
    23% with randomly-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** ***(Tash 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

	Control	+ PTX
Cycles	**30**	**47**
Search time/cycle (min)	**120**	**30**
Cycles with motile sperm	**0**	**45**
Fertilization rate (%)	**50.9[a]**	**66.0[a]**
Clin. pregnancy rate (%)	**26.7**	**38.3**

2. Exposure to motility enhancers

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

	HOST	PTX
Cycles	**25**	**25**
MII oocytes	**336**	**311**
Fertilization rate (%)	**41.1[a]**	**62.1[a]**
Cleavage rate (%)	**86.2**	**89.1**
Clin. pregnancy rate (%)	**16[b]**	**32[b]**

[a,b] $P<0.005$

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 30.3% 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**

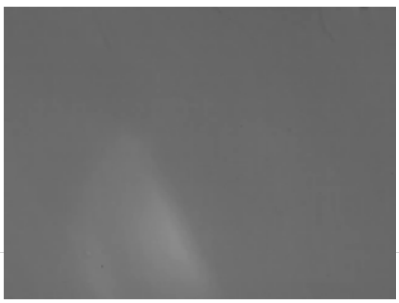
4. Laser-assisted selection (LAISS)

- **First described by *Aktan et al. 2004***
- **Principle: single laser schot 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 HOST
    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)**

4. Laser-assisted selection (LAISS)



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-ejaulated sperm

Comparison MTT and LAISS in UZ Brussel

	MTT		LAISS		
Cycles	23		29		
Oocytes injected	143		248		
	Motile	MTT immot	Motile	LAISS+	LAISS-
Oocytes injected	49	94	103	135	10
Fertilization rate (%)	46.9	31.9	45.6[a]	28.9[a]	20.0
Embryo transfer	14 cycles (60.9%)		18 cycles (62.1%)		
+ hCG	5 cycles		6 cycles		
	3 cycles (2 children)	2 cycles (2 children)	4 cycles (2 children)	2 cycles (1 child)	0 cycles (0 child)

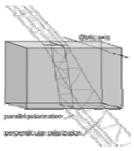
[a] P=0.008

Comparison MTT and LAISS in UZ Brussel

- **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

	Control	Birefringence	P value
Cycles	57	57	
PR per ET	16	35	0.018
Ongoing PR per ET	8	23	0.049

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**

	HOST	birefringence	P value
Cycles	18	20	
Fertilization rate	61.3	75.8	<0.05
Cleavage rate	53.2	68.7	<0.05
Clin pregnancy rate	11.1	45.0	<0.05

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**

References

- Afzelius 1976, Science 193, 317-319
- Aktan et al. 2004, Andrologia 36, 366-369
- Baccetti et al. 2004, J Submicrosc Cytol Pathol 36, 333-339
- Barros et al; 1997, Hum Reprod 12, 1227-1229
- Casper et al. 1996, Fertil Steril 65, 972-976
- Cayan et al. 2001, Fertil Steril 76, 612-614
- De Oliveira et al. 2004, Hum Reprod 19, 262-265
- Eliasson et al. 1977, N Engl J Med 297, 1-6
- Emiliani et al. 2000, Hum Reprod 15, 2371-2374
- Ghosh et al. 2012, Andrologia xx, 1-5
- Gianaroli et al. 2008, Fertil Steril 90, 104-112
- Gianaroli et al. 2010, Fertil Steril 93, 807-813
- Jeyendran et al. 1984, J Reprod Fertil 70, 219-228
- Kahraman et al. 1997, Hum Reprod 12, 292-293
- Kovacic et al. 2006, J Androl 27, 45-52
- Lecomte et al. 1998, In "Male sterility and motility disorders" eds Hamamah et al., 65-78

References

- Liu et al. 1995, Hum Reprod 10, 2630-2636
- Mangoli et al. 2011, Fertil Steril 95, 631-634
- Montag et al. 1999, Andrologia 31, 49-53
- Montag and Rink 2001, ESHRE PCC Embryology, 19-32
- Nagy et al. 1995, Hum Reprod 10, 1123-1129
- Ortega et al. 2011, Hum Reprod Update 17, 684-692
- Soares et al. 2003, Rev Hosp Clin 58, 250-253
- Tash and Means 1983, Biol Reprod 28, 75-104
- Terriou et al. 2000, J Assist Reprod Genet 17, 194-199
- Tournaye et al. 1993, Hum Reprod 8, 1475-1480
- Tournaye et al. 1994, Hum Reprod 9, 1903-1908
- Tournaye te al. 1996, Fertil Steril 66, 331-334
- Tournaye et al. 1998, In "Modern ART in the 2000s" eds Ombelet et al., 157-162
- Vandervorst et al. 1997, Hum Reprod 12, 2429-2433
- Verheyen et al; 1997, Hum Reprod Update 3, 195-203
- Yatsenko et al. 2010, J Androl 31, 34-44
- Yovich et al. 1988, Fertil Steril 50, 179-181
- Zeyneloglu et al. 2000, Hum Reprod 15, 853-856

No sperm today.
Unexpected azoospermia at OPU

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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).

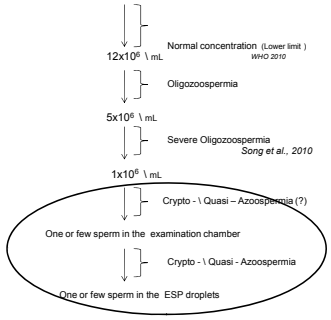
Ron-El et al. , 1997

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



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 azoopermia

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 agents

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, immotile 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 SH et al., 2010

Meaning,

Urgent TESE is not a treatment with which sperm presence is guarantied, also not in crypto azoospermia.

,

The probability crypto azoospermic to turn into azoospermia

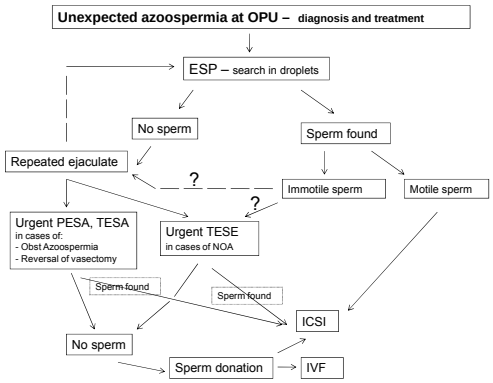
Out of 39 patients with severe non obstructive azoospermia:
< $5x10^6$ \ mL

In a 42 months follow up ;

7 (18%) became crypto-azoospermic
average count 0.1 $x10^6$ \ mL

5 (13%) became azoospermic
was confirmed in ≥2 centrifuged specimens

Song SH et al., 2010



Conclusions:

- Unexpected azoospermia on OPU day is a rare occurrence
- Diagnosis should be confirmed by ESP
- Repeated ejaculation may solve the problem
- Urgent PESA\TESA 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.

2012

Although DNA fragments maybe present , they have no effect on pregnancy rates

לבדוק בספרוף *Collins et al., 2008*

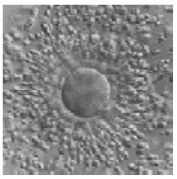
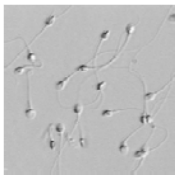
References

- Ron-El R., Strassburger D., Friedler S., Komarovsky D., Bern O., Soffer Y., Raziel A. Extended sperm preparation: an alternative to testicular sperm extraction in non-obstructive azoospermia. Hum. Reprod. 12(6):1222-1226, 1997.
- Nnatu SN, Giwa-Osagie OF, Essien EE. Effect of repeated semen ejaculation on sperm quality. Clin Exp Obstet Gynecol. 1991;18(1):39-42.
- World health Organization Laboratory: Manual for the Examination of Human Semen, 4th ed 1999: Cambridge University Press.
- Tyler JP, Crocket NG, Driscoll GL. Studies of human seminal parameters with frequent ejaculation. I. Clinical characteristics. Clin Repord Fertil 1982; 1: 273-285.
- Song SH, Bak CH, Lim JJ, Yoon TK, Lee DR, Kwon SW. Natural course of severe oligozoospermia in infertile male: influence on future fertility potential. J Androl 2010; 31:536-539.

References

- Meniru GI, Forman RG, Craft IL. Utility of percutaneous epididymal sperm aspiration in situatios of unexpected obstructive azoospermia. Hum Reprod 1997; 12:1013-1014.
- Fisch B, Kaplan-Kraicer R, Amit S, Ovadia J, Tadir Y. The effect of preinsemination interval upon fertilization of human oocytes in vitro. Hum Reprod. 1989; 4:954-6.
- Pellestor F, Girardet A, Andreo B. Effect of long abstinence periods on human sperm quality. Int J Fertil Menopausal Stud. 1994; 39:278-282.
- Cooper TG, Noonan E, von Echardstein S etc., World Health Organization reference values for human semen characteristics. Hum Reprod 2010; 16:231-240.

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

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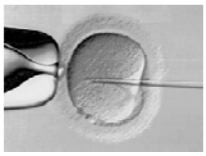
Disclosure

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

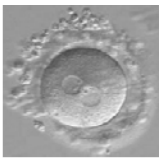
However, total failure of fertilization still occurs in some cases, such as globozoospermia , teratozoospermia, immotile spermatozoa, and even unexplained cases

Rybouchikin AV. et al., 1997, Plachot M. et al., 2002, Kihaile PE. Et al., 2003, Fishel S. et al., 2000

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,

Fertilization rate after ICSI



1- Oocyte "the beauty" and sperm "the beast" quality.

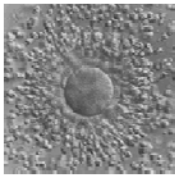
2- ICSI technique itself .

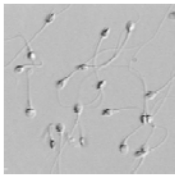
3- Tissue culture conditions in the IVF lab.

Improving fertilization after ICSI

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

Mansour,et al.,2008, Mansour,et al., 2009

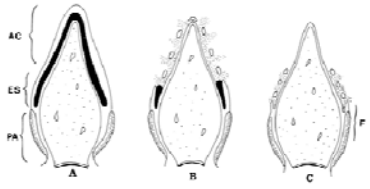




Many hours elapse between ejaculation and in vivo fertilization. During this time, sperm capacitation and the acrosome reaction occur. These crucial steps in gamete interaction allow the penetration of the zona pellucida and fusion with the oocyte membrane.

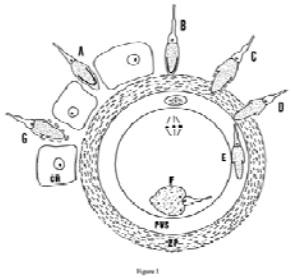
Yanagimachi R. The Physiology of Reproduction. 2nd edn, Raven Press, New York, 1994
Wassarman P.M. exocytosis, and fusion. Cell. 1999

Sperm Acrosome Reaction Diagram (Sathanthan et al., 1993)



A) Acrosome-intact sperm head.
B) Partially acrosome-reacted sperm head
C) Fully acrosome-reacted sperm head
AC = acrosome cap , ES = equatorial segment
F = fusogenic region , PA = post acrosomal segment

Diagram of sperm penetration through egg vestments and sperm incorporation

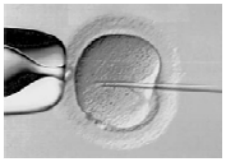


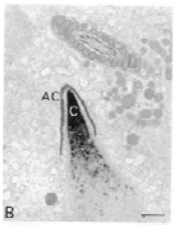
Sathanthan et al., 1993

The ability of spermatozoa to undergo a normal acrosome reaction and the rate of this reaction, are important indicators of fertilizing ability.

Takahashi K, 1992, Makkar G, 2003

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

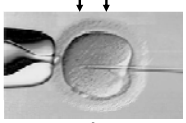




However, the introduction of an acrosome intact sperm into the ooplasm by ICSI seems to physically disturb sperm chromatin decondensation

Katayama M. et al.,2002

Induction of an artificial acrosome reaction



increased fertilization rates and accelerated pronucleus formation

Lacham-Kaplan O. 1995, Lee DR. 1997

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.

Fenichel P. 1991, Fenichel P. 1995, Schill WB, 1988

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.

Mansour,et al.,2008

Design

Semen Processing

⇓⇓

Sperm incubation at 5% Co_2 and 37°C for:

⇓⇓

EM ⟵ *a) one hour* ⟶ *ICSI*

EM ⟵ *b) Three hours* ⟶ *ICSI*

EM ⟵ *c) Five hours* ⟶ *ICSI*

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

Sperm incubation time before ICSI	1 h group	3 h group	5 h group
ICSI cycles	83	87	81
Age year (mean ± SD)	32.34 ± 2.52	32.05 ± 3.35	33.01 ± 1.34
Oocytes retrieved (mean ± SD)	810 (9.9 ± 2.5)	840 (10.2 ± 3.2)	795 (10.04 ± 1.55)
M2 oocytes (mean ± SD)	648 (8.5 ± 4.1)	672 (7.9 ± 2.5)	640 (8.2 ± 2.3)
2 PN oocytes (mean ± SD)	453 (4.6 ± 0.5)	498 (6.5 ± 1.9)	428 (5.9 ± 1.5)
Fertilization rate	70%	74%	67%
Embryos per ET (mean ± SD)	3.0 ± 0.43	2.99 ± 0.63	3.02 ± 0.24
Implantation rate	22.52%	21.42%	20.55%
Clinical pregnancies pregnancy rate	46 (54.8%)	51 (56.7%)	43 (52.4%)

(Mansour et al., 2008)

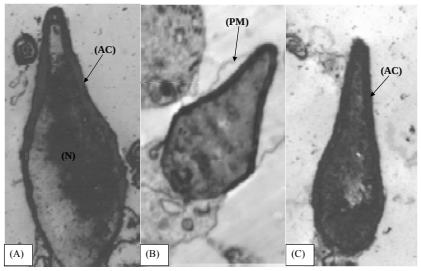
Table 2. Rate of acrosome reaction in relation to the sperm incubation time

Sperm incubation time	Semen parameters (mean ± SD)	Total umber of sperm heads studied	Number of sperm heads with acrosomal reaction	Rate of acrosomal reaction
1 hour	Count 7.5 ± 2.4X10^6/mL Motility 11.2 ± 5.5% Abnormal forms 79.5 ± 11.2%	308	79	25.6%[a]
3 hours	Count 8.2 ± 1.2X10^6/mL Motility 10.5 ± 6.4% Abnormal forms 80.7 ± 9.5%	298	122	40.9%[b]
5 hours	Count 9.2 ± 6.5X10^6/mL Motility 12.3 ± 5.8% Abnormal forms 78.6 ± 9.8%	251	171	68.2%

[a] *Significant difference as compared to 3h group [O.R = 0.63, 95% CI = 0.45 to 0.87, P = 0.005]. Significant difference as compared to 5h group [O.R = 0.22, 95% CI = 0.15 to 0.31, P = 0.0001].*
[b] *Significant difference as compared to 5h group [O.R = 0.6, 95% CI = 0.45 to 0.8 P = 0.0005].*

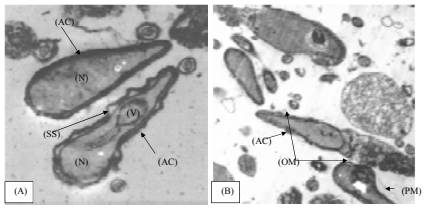
(Mansour et al., 2008)

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 nonclear subacrosomal space. The chromatin is compact and dense within the nucleus (N) with no visible vacuoles (magnification X 31,760), B) An acrosome-intact sperm head with the plasma membrane (PM) swollen away from the acrosome (X 26467), C) A sperm head with ruptured plasma membrane and intact nonreacted acrosome (AC) (magnification X 31,760).



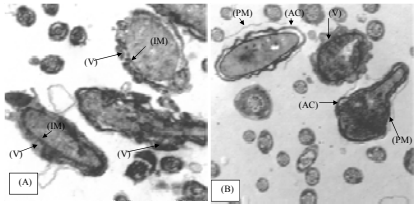
(Mansour et al., 2008)

Fig. 2: Transmission electron microscopic photomicrographs 3-hour group. (A) Two sperm heads, the upper head has a ruptured plasma membrane, intact nonreacted 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).



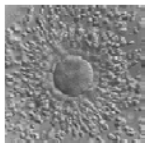
(Mansour et al., 2008)

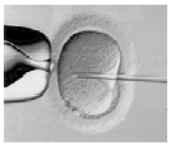
The electron microscopic results in our study showed that the rate of acrosome reaction was time dependent, with a maximum of 5 hours. However, the fertilization rate was the highest when the spermatozoa were incubated for 3 hours.

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

Rybouchibin 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

Rybouchikin 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.

Yanagida 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.*

Mansour,et al.,2009

Sibling oocytes from each patient were randomly divided into two groups:

1- Electroactivated group

2- control group

Mansour,et al.,2009

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.

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.

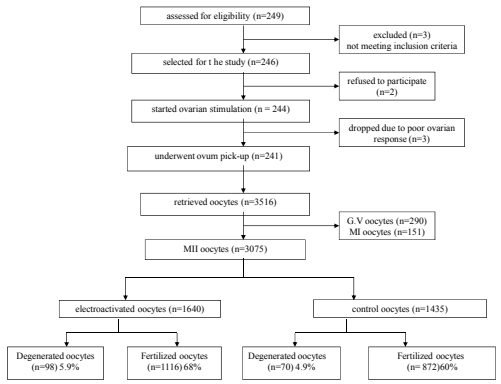
The electrically stimulated oocytes immediately were transferred back to the tissue culture media to be rinsed then they were incubated under oil in 5% Co_2 in air, at 37ºC.

Table 1. Results of oocyte electroactivation in 10 ICSI cycles for patients with previous total failure of fertilization

Case number	Oocytes Retrieved	MII oocytes	2 PN oocytes	Embryos NO. of transferred	NO. of embryos Cryopreserved	Pregnancy results
1	13	8	5	3	--	-ve
2	4	4	1	1	--	-ve
3	18	10	2	2	--	-ve
4	12	7	3	3	--	single
5	14	8	3	2	--	-ve
6	12	6	2	1	--	-ve
7	4	4	2	2	--	-ve
8	12	9	7	3	4	single
9	6	5	2	2	--	single
10	8	5	5	3	2	single
Total	103	66	31	22	6	4 healthy babies

Note: -ve = negative

Mansour,et al.,2009



Mansour,et al.,2009

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

Parameter	Electroactivated oocytes	Control oocytes
No. (mean ± SD) of metaphase II oocytes	1,640 (6.8 ± 2.48)	1,435 (5.95 ± 29)
No. (mean ± SD) of 2-pronuclear oocytes	1,116 (4.63 ± 2.3)	872 (3.62 ± 1.96)
Fertilization rate (%)[a]	68	60
No. (mean ± SD) of Degenerated oocytes	98 (1.73 ± 1.2)	70 (1.39 ± 0.79)
Degeneration rate (%) [b]	5.9	4.9%

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

Parameter	**Embryos for ET derived from electro-activated group**	**Embryos for ET derived from control group**	**Embryos for ET derived from both groups**
No. of ET procedures	34	69	138
No. of clinical pregnancies	15 (44)	33 (48)	64 (46.4)
No. of miscarriage	3 (20)	3 (9)	6 (9.4)
Ectopic pregnancy	----	1	----
No. of deliveries	12 (4 sets of twins, and 8 singletons, totaling 16 healthy babies, [9♀ + 7♂)]	29 (6 sets of twins and 23 singletons, totaling 34 healthy babies, [14♀ + 20♂] and 1 stillbirth)	58 (1 set of triplets, 10 twins, and 47 singletons, totaling 70 healthy babies, [30♀ + 40♂])

Mansour,et al.,2009

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

Mansour,et al.,2009

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.

Mansour,et al.,2009

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.*

References

- *Devroey P, Van Steirteghem A. A review of ten years experience of ICSI. Hum Reprod Update 2004;10:19–28.*
- *Ebner T, Moser M, Sommergruber M, Jesacher K, Tews G. Complete oocyte activation failure after ICSI can be overcome by a modified injection technique. Hum Reprod 2004;19:1837–41.*
- *Fenichel P, Donzeau M, Cervoni F, Menezo Y, Hsi BL. Expression of complement regulatory proteins on human eggs and preimplantation embryos. Am J Reprod Immunol 1995;33:155–64*
- *Fenichel P, Donzeau M, Farahifar D, Basteris B, Ayraud N, Hsi BL. Dynamics of human sperm acrosome reaction: relation with in vitro fertilization. Fertil Steril 1991;55:994–9.*
- *Fishel S, Aslam I, Lisi F, Rinaldi L, Timson J, Jacobson M, et al. Should ICSI be the treatment of choice for all cases of in-vitro conception? Hum Reprod 2000;15:1278–83.*
- *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: 2237–41.*
- *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 2005;20: 2237–41.*

References (2)

- *Katayama M, Koshida M, Miyake M. Fate of the acrosome in ooplasm in pigs after IVF and ICSI. Hum Reprod 2002;17:2657–64.*
- *Kihaile PE, Misumi J, Hirotsuru K, Kumasako Y, Kisanga RE, Utsunomiya T. Comparison of sibling oocyte outcomes after intracytoplasmic sperm injection and in vitro fertilization in severe teratozoospermic patients in the first cycle. Int J Androl 2003;26:57–62.*
- *Lacham-Kaplan O, Trounson A. Intracytoplasmic sperm injection in mice: increased fertilization and development to term after induction of the acrosome reaction. Hum Reprod 1995;10:2642–9.*
- *Lee DR, Lee JE, Yoon HS, Roh SI. Induction of acrosome reaction in human spermatozoa accelerates the time of pronucleus formation of hamster oocytes after intracytoplasmic sperm injection. Fertil Steril 1997;67: 315–20.*
- *Mahutte NG, Arici A. Failed fertilization: is it predictable? Curr Opin Obstet Gynecol 2003;15:211–8.*
- *Makkar G, Ng EH, Yeung WS, Ho PC. The significance of the ionophore-challenged acrosome reaction in the prediction of successful outcome of controlled ovarian stimulation and intrauterine insemination. Hum Reprod 2003;18:534–9.*

References (3)

- *Mansour R, Fahmy I, Tawab NA, Kamal A, El-Demery Y, Aboulghar M, Serour G. Electrical activation of oocytes after intracytoplasmic sperm injection: a controlled randomized study. Fertil Steril. 2009 Jan;91(1):133-9. Epub 2008 Oct 1.*
- *Mansour RT, Serour MG, Abbas AM, Kamal A, Tawab NA, Aboulghar MA, Serour GI. The impact of spermatozoa preincubation time and spontaneous acrosome reaction in intracytoplasmic sperm injection: a controlled randomized study. Fertil Steril. 2008 Sep;90(3):584-91. Epub 2008 Mar 4.*
- *Plachot M, Belaisch-Allart J, Mayenga JM, Chouraqui A, Tesquier L, Serkine AM. Outcome of conventional IVF and ICSI on sibling oocytes in mild male factor infertility. Hum Reprod 2002;17:362–9.*
- *Rybouchikin AV, Van der Straetem F, Quatacker J, De Sutter P, Dhont M. Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. Fertil Steril 1997;68:1144–7.*
- *Rybouchikin AV, Van der Straetem F, Quatacker J, De Sutter P, Dhont M. Fertilization and pregnancy after assisted oocyte activation and intracytoplasmic sperm injection in a case of round-headed sperm associated with deficient oocyte activation capacity. Fertil Steril 1997;68:1144–7.*

References (4)

- *Schill WB, Topfer-Petersen E, Heissler E. The sperm acrosome: functional and clinical aspects. Hum Reprod 1988;3:139–45.*
- *Takahashi K, Wetzels AM, Goverde HJ, Bastaans BA, Janssen HJ, Rolland R. The kinetics of the acrosome reaction of human spermatozoa and its correlation with in vitro fertilization. Fertil Steril 1992;57: 889–94.*
- *Wassarman PM. Mammalian fertilization. molecular aspects of gamete adhesion, exocytosis, and fusion. Cell 1999;22,96:175–83.*
- *Yanagida K, Katayose H, Yazawa H, Kimura Y, Sato A, Yanagimachi H, et al. Successful fertilization and pregnancy following ICSI and electrical oocyte activation. Hum Reprod 1999;14:1307–11.*
- *Yanagida K. Complete fertilization failure in ICSI. Hum Cell 2004;17: 187–93.*
- *Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill J, eds. The physiology of reproduction. 2nd ed. New York: Raven Press, 1994: 189–317.*

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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

eshre European Society of Human Reproduction and Embryology

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 patrilinear 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

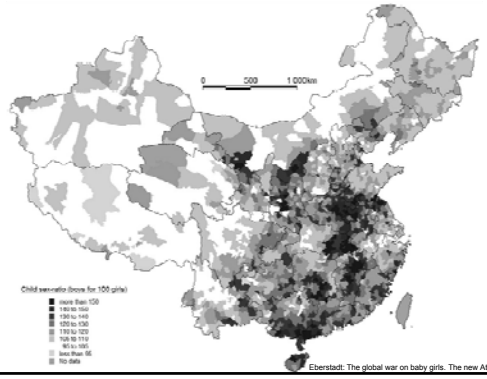
Secundary Sex Ratio

Worldwide: 1.07 males per female
European Union: 1.06

• Azerbeijan	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		

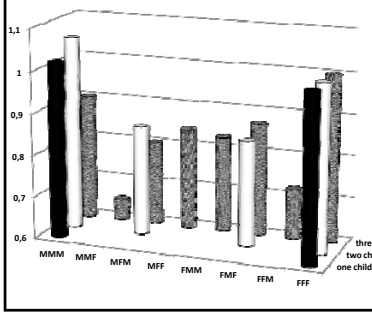
CIA Factbook World 1012

Sex ratio imbalance in China



Eberstadt: The global war on baby girls. The new Atlantis, 2012

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



Gender combination (no. of families)	Fertility rate ratio (95% CI)
One-child families	
M (186 637)	1.02 (1.01–1.02)
F (176 736)	1.00
Two-children families	
MM (54 466)	1.06 (1.03–1.09)
MF (51 131)	0.86 (0.83–0.88)
FM (50 998)	0.85 (0.82–0.87)
FF (48 220)	1.00
Three-children families	
MMM (6012)	0.90 (0.82–0.99)
MMF (5864)	0.65 (0.58–0.72)
MFM (4843)	0.80 (0.72–0.89)
MFF (4994)	0.84 (0.73–0.93)
FMM (4755)	0.83 (0.75–0.93)
FMF (4458)	0.87 (0.78–0.97)
FFM (5146)	0.72 (0.65–0.80)
FFF (4861)	1.00

[a]Reference category.
M = male, F = female.

R. Jacobsen et al. Hum. Reprod. 1999

Timing (Shettles) method

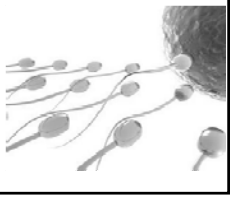
Y-bearing sperm is smaller, faster, more fragile

Intercourse close to ovulation favours boys

For a girl: Intercourse 2 – 3 days before ovulation

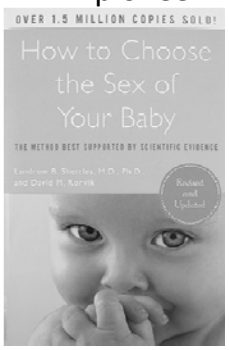
Supposed success rate: 75%

Very popular, widely applied for over 40 years



L. Shettles, Int. J. Gynaecol. Obstet. 1970

IVF pioneer Landrum B. Shettles



1970 – 2006 (6th edition)

Landrum B. Shettles 1909 - 2003

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

A. Wilcox et al. N. Engl. J. Med. 1995

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

Francois Papa

Multicenter experience:
500 women participated
75 – 85% were succesful

Success depended largely on how strictly participants adhered to the diet

No objective quantification of compliance

Stolkowski and Lorrain, Int. J. Gycaecol. Obstet. 1980

Previous research diet method

Researchers	Year	n	Succesful	Success Rate (%)
Stolkowski & Lorrain	1980	260	212	82
Stolkowski & Choukroun	1981	47	40	85
Papa et al.	1983	58	45	78
Jeambrun	1989	61	46	75
Devaure et al.	1989	72	58	81
Total		**498**	**401**	**81**

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

P. Hudson, R. Buckley, Pract. Midwife 2000
Mathews et al, Proc. R. Soc. B :Biol. Sci. 2008

In my practice

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

Reasons for wanting a boy at GC

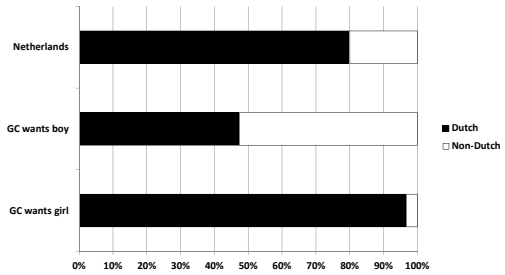
- Fathers find it important to have a son
- Male heir, family name
- Successor for firm or farm
- Having a son is important for Muslims
- Fathers can do man-things with a son
- Loss of a son earlier

Reasons for wanting a girl at GC

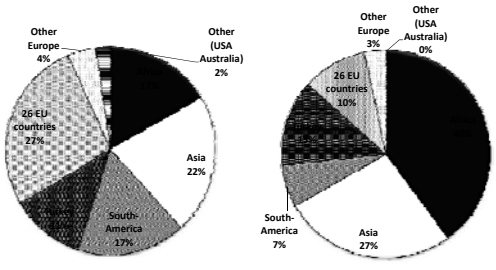
- Having a daughter is important for mothers
- Mother expects a closer relationship than with boy; identification with own gender
- Easier to handle, sweeter
- Mixed family, family balancing
- Loss of a daughter in pregnancy or as a baby

A son is a son until he gets himself a wife,
but a daughter is your daughter for the rest of your life.

Ethnic background



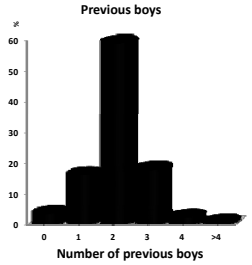
Composition of non-Dutch citizens



Netherlands GC clients for a boy

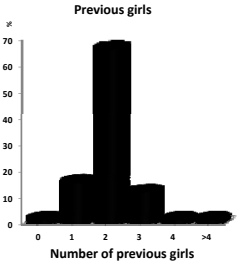
Break-down of GC clients

85% wants a girl



3% wants a second girl
3% has lost a girl

15% wants a boy

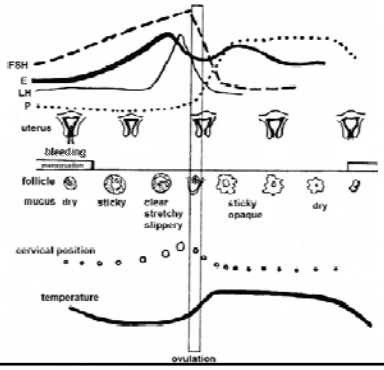


7% wants a second boy
5% has lost a boy

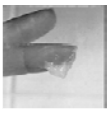
Treatment CG: Timing

- 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

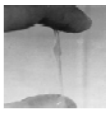
Hormonal changes during cycle



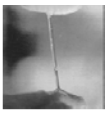
Assessment of cervical mucus



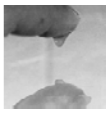
Opaque, sticky



Fertile, stretchy



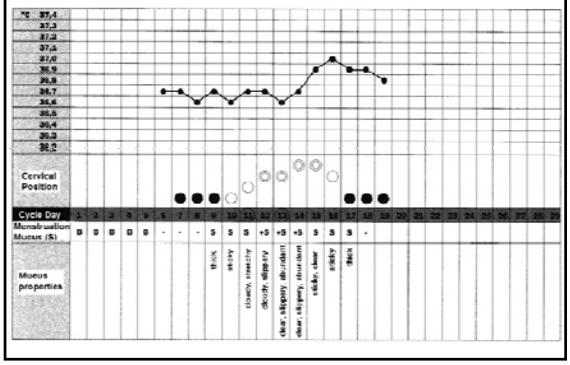
Clear, stretchy



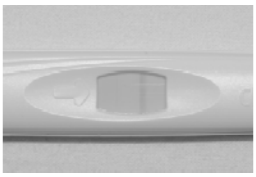
After ovulation

From: Billings&Westmore: The Billings Method, 1980

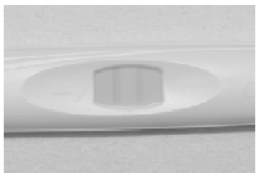
Temperature Chart



Ovulation Tests



Negative



Positive

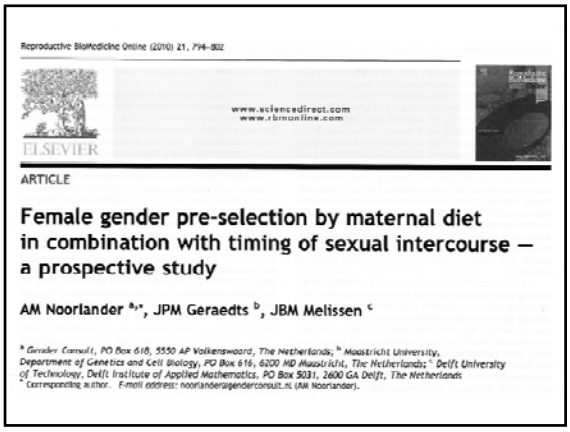
CG Treatment: Diet

- 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 pregancy
- 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



Reproductive BioMedicine Online (2010) 21, 794-802

www.sciencedirect.com
www.rbmonline.com

ELSEVIER

ARTICLE

Female gender pre-selection by maternal diet in combination with timing of sexual intercourse – a prospective study

AM Noorlander [a,*], JPM Geraedts [b], JBM Melissen [c]

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* Corresponding author. E-mail address: noorlander@genderconsult.nl (AM Noorlander).

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

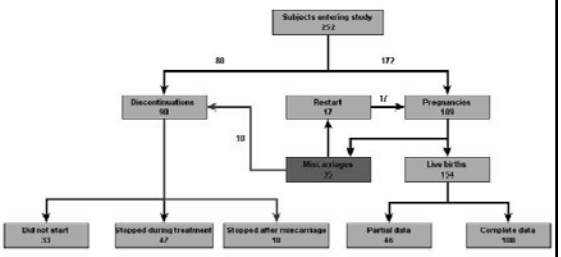
Study population

- GC clients with a preference for a girl
- Period 2001 – 2011
- Healthy couples
- Age mother: 34.0 $\pm$ 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

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

Partial data

Reason	n
No post-pregnancy blood sample drawn	19
Diet was not started	11
Diet shorter than 9 weeks	10
No ovulation tests applied	6
Total (23 girls, 23 boys)	46

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

	Prediction rule satisfied	Prediction rule not satisfied	Total without prediction rule
Reference group	91% 10♀, 1♂ n = 11	24% 4♀, 13♂ n = 17	50% 14♀, 14♂ n = 28
Validation group	79% 26♀, 7♂ n = 33	40% 19♀, 28♂ n = 47	56% 45♀, 36♂ n = 80
Total research group	**82%** **36♀, 8♂** **n = 44**	**36%** **23♀, 41♂** **n = 64**	55% 59♀, 49♂ n = 108

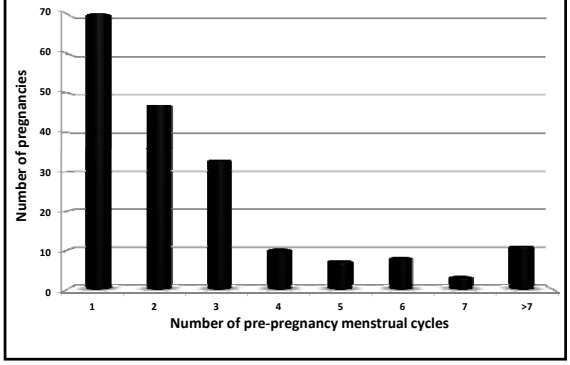
Results

	Timing favouring girl	Timing favouring boy	Total
Diet correct	**82%** **36 ♀, 8 ♂** **n = 44**	50% 5 ♀, 5 ♂ n = 10	76% 41 ♀, 13 ♂ n = 54
Diet incorrect	36% 15 ♀, 26 ♂ n = 41	23% 3 ♀, 10 ♂ n = 13	33% 18 ♀, 36 ♂ n = 54
Total	60% 51 ♀, 34 ♂ n = 85	34% 8 ♀, 15 ♂ n = 23	55% 59 ♀, 49 ♂ n = 108

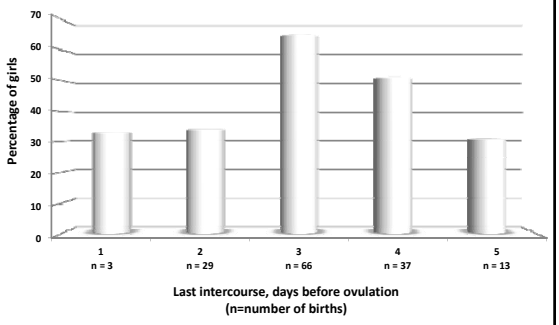
Group satisfying the prediction rule

- 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

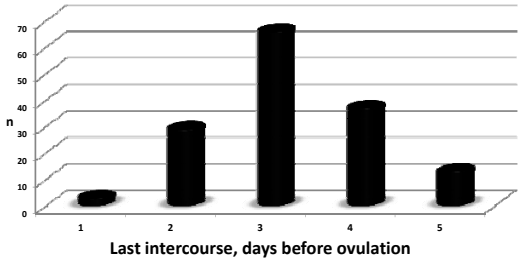


Success rate of timing



The succes rate for 3 days, 65%, is significant, p = 0.0015

Compliance of timing



Compliance with timing = 78%

Success rate of diet

	n	Girls	Success
Reference group	11	10	91%
Validation group	46	33	72%
Total	57	44	77%

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

	Before diet	After 5 weeks	End of diet	Reference range
Na^+	141.6 ± 2.6	139.9 ± 2.1	139.2 ± 2.7	135 – 150
K^+	4.40 ± 0.48	4.40 ± 0.41	4.38 ± 0.42	3.6 – 5.4
Ca^{2+}	2.38 ± 0.11	2.41 ± 0.09	2.40 ± 0.11	2.1 – 2.7
Mg^{2+}	0.89 ± 0.13	0.91 ± 0.13	0.90 ± 0.12	0.70 – 1.10

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

- **Billings, EL, Westmore, A, 1980.** The Billings Method, controlling fertility without drugs or devices. 4th Ed. Gordon & Gotch.
- **Bolet, GL, Guenguen, P, Dando, P, Ollivier, L, 1982.** Influence of mineral diet of the sow on the sex ratio of newborn. Reprod. Nutr. Dev. 22, 1073–1081.
- **CIA Factbook World 1012.** https://www.cia.gov/library/publications/the-world-factbook/
- **Devaure, N, Dabadie, H, Paccalin, J, 1989.** Influence des apports nutritionnels dans le determinisme du sexe. Diet. Med. 2,155–163.
- **Duc, M, 1977.** De l'influence des apport nutritionnels en ions K+,Na+, Ca2+, Mg2+ sur la sex-ratio chez l'homme. Ph.D. thesis, Université de Paris-Val-de-Marne, 56p.
- **Eberstadt, N, 2012** The global war on baby girls. The new Atlantis, http://www.thenewatlantis.com/publications/the-global-war-against-baby-girls
- **Herbst, C, 1935.** Untersuchungen zur Bestimmung des Geschlechts:II. Die Abhängigheit des Geschlechts vom Kaliumgehalt desumgebenden Mediums bei Bonella viridis. Roux Arch. Ent. Org. 132, 578–599.
- **Hudson, P, Buckley, R, 2000.** Vegetarian diets: are they good for pregnant women and their babies? Pract. Midwife 3, 22–23.
- **Jacobsen, R, Møller, M, Engholm, G, 1999.** Fertility rates in Denmark in relation to the sexes of preceding children in the family. Hum. Reprod. 14, 1127–1130.
- **Jeambrun, P, 1989.** Escolha pré-concepcional do sexo por meio de dieta. Servir 38, 179–182.
- **Lorrain, J, 1975.** Pre-conceptional sex selection. Int. J. Gynecol. Obstet. 13, 127–130.
- **Mathews, F, Johnson, PJ, Neil, A, 2008.** You are what your mother eats: evidence for maternal preconception diet influencing foetal sex in humans. Proc. R. Soc. B: Biol. Sci. 275, 1661–1668.
- **Noorlander, AM, Geraedts, JPM, Melissen, JBM, 2010:** Female gender pre-selection with timing of sexual intercourse – a prospective study. RBMOnline 21, 794 – 802.
- **Papa, F, Henrion, R, Breart, G, 1983.** Sélection pré-conceptionelle du sexe par la methode ionique. J. Gynecol. Obstet. Biol. Reprod. (Paris) 12, 415–422.
- **Shettles, LB, 1970.** Factors influencing sex ratios. Int. J. Gynecol. Obstet. 8, 643–647.
- **Shettles, LB, Rorvik, DM, 2006.** How to choose the sex of your baby. 6th ed. Broadway Books.
- **Stolkowski, J, 1967.** Influence possible de la nutrition minérale sur la répartition des sexes chez la vache: une enquête rétrospective. C. R. Acad. Sci. D (Paris) 265, 1059–1062.
- **Stolkowski, J, Choukroun, J, 1981.** Preconception selection of sex in man. Isr. J. Med. Sci. 17, 1061–1067.
- **Stolkowski, J, Lorrain, J, 1980.** Preconceptional selection of fetal sex. Int. J. Gynecol. Obstet. 18, 440–443.
- **Vahidi, AR, Sheikhha, M.H., 2007**. Comparing the effects of sodium and potassium diet with calcium and magnesium diet on sex ratio of rats' offspring. Pak. J. Nutr. 6, 44–48.
- **Wilcox, AJ, Weinberg, CR, Baird, DD, 1995.** Timing of sexual intercourse in relation to ovulation. N. Engl. J. Med. 333, 1517–1521.

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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