European Society of Human Reproduction and Embryology

COURSE 2

"To what extent do male and female gametes influence embryo development?"

Special Interest Groups
Embryology
Andrology

1 July 2007
Lyon, France

PRE-CONGRESS COURSE 2

Special Interest Groups Embryology and Andrology
"To what extent do male and female gametes influence embryo development"

CONTENTS

PRE-CONGRESS COURSE 2 - PROGRAM

Joint SIG Embryology and SIG Andrology

"To what extent do male and female gametes influence embryo development?"

Course co-coordinators: Etienne Van den Abbeel (SIG Embryology), Dominique Royère (SIG Embryology), Lars Björndahl (SIG Andrology), TBA (SIG Andrology)

Course description: a basic course on paternal and maternal contributions to human embryo development in-vitro

Target audience: clinical embryologists and andrologists and clinicians

Program

Topic I: Paternal contributions to human embryo development

09.00 – 09.30: Paternal influences on embryo development: from pronucleus to genomic activation - ***J.P. Renard (F)***
09.30 – 10.00: Is it useful to perform chromatin assays in ART: a critical assessment - ***J Tesarik (E)***
10.00 – 10.15: Discussion

10.15 – 10.45: Coffee break

Topic II: Maternal contributions to human embryo development

10.45 – 11.15: Oocyte – cumulus dialog and mammalian embryo development - ***D. Albertini (USA)***
11.15 – 11.45: Strategies to improve clinical in-vitro maturation of oocytes - ***J Smitz (B)***
11.45 – 12.00: Discussion

12.00 – 13.00: Lunch

13.00 – 14.00: Business meeting of the Special Interest Group Embryology

Topic III: Sperm and egg interactions

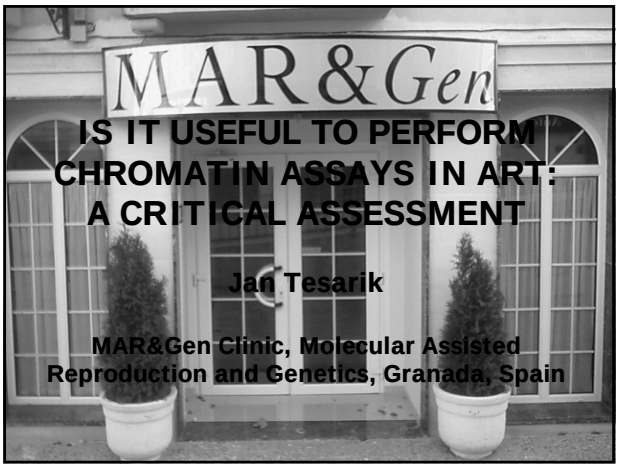
14.00 – 14.30: Sperm - egg fusion, ICSI and egg activation: what's new? – ***J. Evans (USA)***
14.30 – 15.00: Optimizing in-vitro fertilization systems - ***D Mortimer (CND)***
15.00 – 15.15: Discussion

15.15 – 15.45: Coffee break

Topic IV: How to improve embryo selection?

15.45 – 16.15: Selecting sperm - ***D Sakkas (USA)***
16.15 – 16.45: Selecting oocytes - ***U Eichenlaub-Ritter (D)***
16.45 – 17.15: Selecting embryos - ***F Guérif (F)***
17.15 – 17.30: Discussion

17.30 - 18.30: *Business meeting of the Special Interest Group Andrology*



IS IT USEFUL TO PERFORM CHROMATIN ASSAYS IN ART: A CRITICAL ASSESSMENT

Jan Tesarik

MAR&Gen Clinic, Molecular Assisted Reproduction and Genetics, Granada, Spain

Etiology of sperm nuclear DNA damage

- **Necrosis**
- **Apoptosis (programmed cell death - PCD)**
- **Abortive apoptosis or oxidative cell damage without the activation of apoptotic cell signalling pathway**
- **Abnormal sperm chromatin condensation**

Necrosis evaluation

- Viability tests
  - supravital eosin staining
  - hypo-osmotic swelling test

Physical and biological changes in apoptosis

- Shrinkage, which detaches the cell from adjacent cells
- Traslocation of PS from the inner cytoplasmic membrane to the outer surface.
- Release of cytochrome C and Apoptosis Inducing Factor (AIF) into the cytoplasm from the mitochondrial (propapoptotic proteins decrease the mitochondrial transmembrane potential)
- Uniform aggregation of chromatin along the membrane.
- Fragmentation of the nuclear DNA into lengths of ~200 bp.
- Degradation of the cell into apoptotic bodies and phagocytosis by neighboring cells or macrophages.

Wyllie AH et al. 1980 and Vaux DL et al. 1996

Techniques for determination of sperm DNA fragmentation

- **SCDA / SPERM CHROMATIN DISPERSION ASSAY**
- **AO: Acridine orange + microscopy**
- **SCSA: Acridine Orange + Flow cytometry**
- **Comet: Electrophoresis + Fluorescent dye**
- **NT: Nick translation biotinylated UTP**
- **Tunel: UTP binding + Flow cytometry or microscopy**

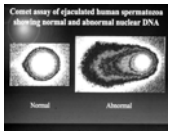
SCSA technique
(Sperm Chromatin Structure Assay)

- **Use of acridine orange and flow cytometry.**
- **Calculation of DNA fragmentation index :DFI (Evenson D et col).**
- **DFI appears correlated with DNA breakage measured by COMET or TUNEL**
- **Fertility prognosis :**
  DFI<15% : good
- **15%<DFI<30%: uncertain**
  DFI>30% : poor (few pregnancies and high pregnancy wastage)
- **Impact on early embryo development**

COMET technique

(Single Cell Gel Electrophoresis : SCGE)

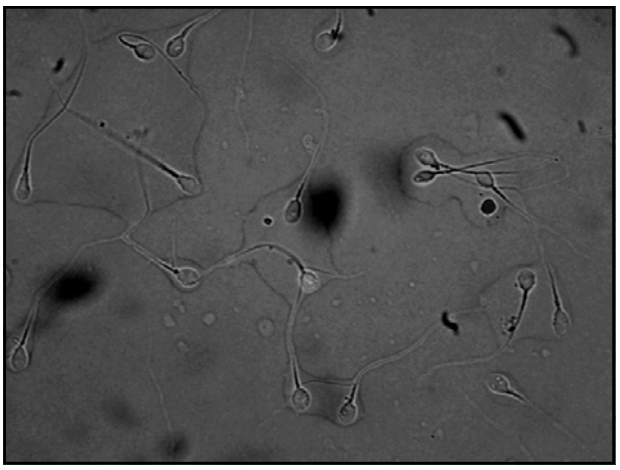
- **Sperm on very thin agarose gel on slide, in situ lysis, electrophoresis, labelling with fluorescent ADN stain.**
- **Fluorescence intencity and length of comet tail are correlated with the extent of DNA strand breaks.**



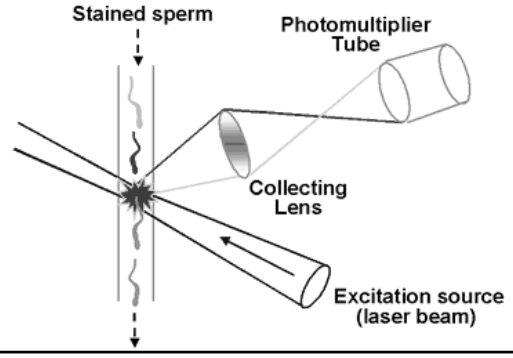
TUNEL technique

TdT-mediated dUTP Nick End Labeling

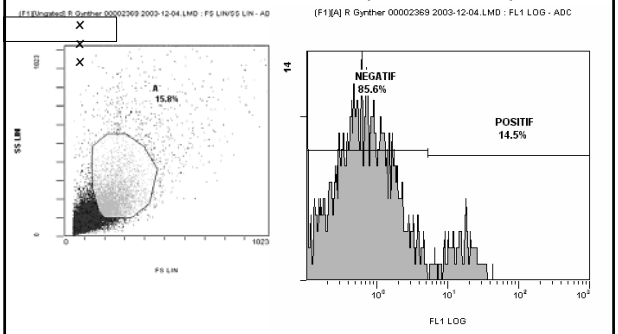
- **Direct techniaue for visualization of DNA fragmentation in cell nuclei.**
- **Incorporation of labeled nucleotides to ends of broken DNA strands by the action of terminal deoxynuceotide transferase**
- **Biotinylated nucleotides + fluorochrome-couples Abs**
- **Fluorescence microscopy or flow cytometry**



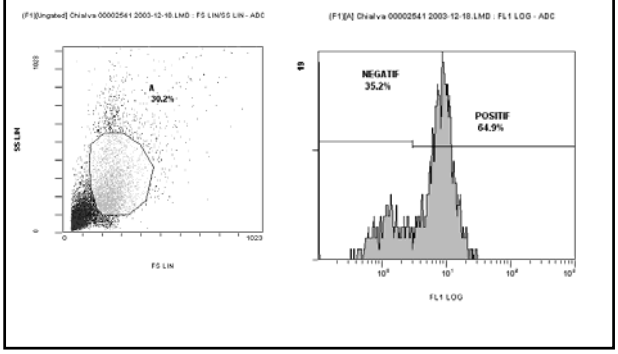
Flow cytometry



TUNEL – flow cytometry



TUNEL – flow cytometry



Apoptosis and spermatogenesis

• Programmed cell death is required for normal spermatogenesis in mammalians (to ensure cellular homeostasis) *Rodriguez et al., 1997*

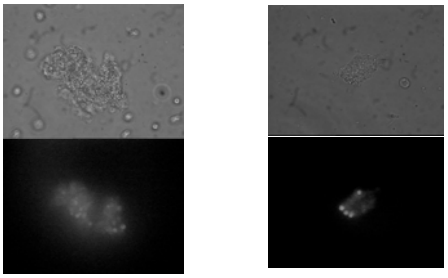
• Enhancement of apoptosis is the result of various testicular and systemic pathologies (maturation arrest, ipospermatogenesis …) *Gandini et al., 2000; Tesarik at al., 1998*

We have analysed the relationship between caspase activation and DNA damage in both germ end Sertoli cells maintained in vitro within explanted segments of the human seminiferous tubules

Tesarik et al., 2002

Programmed Cell Death in Testicular Biopsy

III) Patient whith Sertoli cell only Syndrome



Caspase activity in isolated Sertoli cell. The activity is accumulated in cytoplasmic granules (absent in the nucleous).

- Nuclear DNA fragmentation in ejaculated spermatozoa is due to abortive apoptosis or a nonspecific post-testicular oxidative damage rather than classical apoptosis:
- Diagnostic considerations
- Treatment options

DNA frqgmentation and IVF

There is a negative correlation between TUNEL+ IVF outcome

Sun J.G. et col, Biol. Reprod., 1997

DNA fragmentation and ICSI

Negative correlation between TUNEL+ and fertilization
(Baccetti B et col, Cytol. Pathol., 1996)
(Lopes S et col, Fertil. Steril. , 1998)

Impact of DNA fragmentation on ICSI outcome not found in all studies.
(Host E. et col, Acta.Obstet. Gynecol. Scand., 2000)

DNA **fragmentation** of 104 couples (IVF: 50; ICSI: 54)

- **TUNEL assay**
- **Negative correlation between DNA fragmentation extent and basic sperm parameters**
- **If>10% TUNEL+,↓ fertilization rate**
- **No relationship with embryo grade**
- **No relationship with pregnancy rate in IVF**
- **Impairment of pregnancy rate in ICSI**

Human Reproduction, May 2003
Benchaib et al

Most frequently used thresholds

Technique	Threshold	Clinical context	Reference
TUNEL (microscopy)	12% TUNEL+	IUI	Duran *et al.*, 2002
TUNEL (microscopy)	18% TUNEL+	ICSI	Benchaib *et al.*, 2003
TUNEL (microscopy)	24.3% TUNEL+a	ICSI	Henkel *et al.*, 2003
TUNEL (microscopy)	36.5% TUNEL+a	IVF	Henkel *et al.*, 2003
SCSA (flow cytometry)	27% DFIb	IVF and ICSI	Larson-Cook *et al.*,
SCSA (flow cytometry)	30% DFIb	IVF and ICS	Virro *et al.*, 2004
TUNEL (microscopy)	15% TUNEL+a	ICSI	Greco *et al.*, 2005
TUNEL (flow cytometry)	30% TUNEL+ a	ICSI	Hazout *et al.*, 2006

- **If fragmentation moderated, it can be repaired by oocyte**
  - **Sakkas *et al.*, 1996_;**
  - **Ahmadi and Ng, 1999**
- **If fragmentation high, repair impossible**
  - **Sakkas *et al.* (1996_)**
- **Fragmentation may cause sperm chromatin decondensation failure**

Association with previous failure

	% TUNEL-positive spermatozoaa	
	Group A (first attempts) (n = 343)	Group B (third attempts) (n = 136)
Childbirth	10.3 ± 1.4b	6.9 ± 1.3b
No childbirth	19.2 ± 3.3	17.8 ± 3.4

Effect of female age

	% TUNEL-positive spermatozoaa	
	Group A (patient oocytes) (n = 268)	Group B (donor oocytes) (n = 281)
Female age	34.7 ± 3.2	21.8 ± 1.1
Childbirth	6.4 ± 1.4	11.8 ± 2.9
No childbirth	13.0 ± 2.8	13.7 ± 3.2

- **Treatment options for male infertility due to sperm nuclear DNA fragmentation**

ICSI clinical outcomes: ejaculated versus testicular sperm

Sperm Source	Attempts	Clinical pregnancies	Pregnancy rate	Implantation rate
Ejaculate	**18**	**1**	**5.6%**	**1.8%**
Testis	**18**	**8**	**44.4%**	**20.7%**

Greco et al., Hum.Reprod. 2005

Search for less invasive approaches

- **Oral antioxidant treatment**
- **High-magnification ICSI (HM-ICSI)**

Oral antioxidant treatment

(1g vitamin C, 1 g vitamin E daily, 2 months)

Time of analysis	TUNEL+ sperm (%)
Before treatment	22.1 ± 7.7
	P<0.001
After treatment	9.1 ± 7.2

Greco et al., J. Androl 2005

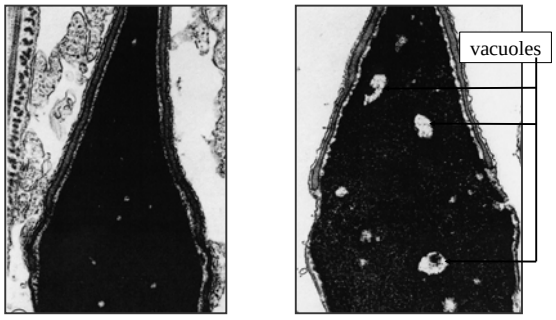
Oral antioxidant before ICSI

(1g vitamin C, 1 g vitamin E daily, 2 months)

Time of attempt	Pregnancy rate	Implantation rate
Before treatment	6.9%	2.2%
	P<0.001	P<0.001
After treatment	48.3%	19.6%

Greco et al., Hum. Reprod. 2005

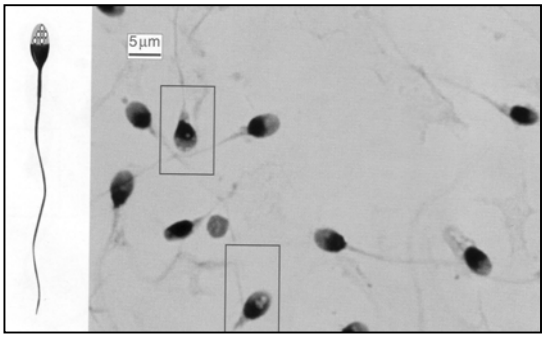
- **High-magnification ICSI (HM-ICSI)**



Well-condensed chromatin **Poorly condensed chromatin**

Bedford et al,1973 (x 46000)

VACUOLES



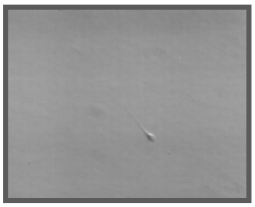
MSOME

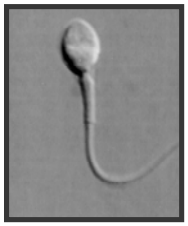
Motile Sperm Organelle Morphology Examination

- **Inverted microscope (Olympus IX70)**
- **Nomarski contrast: x100 immersion lens**
- **Video camera**
- **Total magnification = direct optical magnification (150x) x video coupler magnification (0,99x) x video camera magnification(44,45x) = 6600x**
- **Observation Wilco dish: glass bottom 170µm thick**

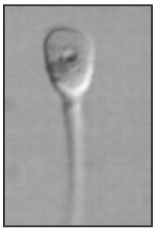
Bartoov et al .2002, J Androl 23:1-8

×400 ×6600





Données AMP EYLAU



Sperm heads with different-sized vacuoles visualized by MSOM

Conventional- vs HM-ICSI in patient with >40% DNA-fragmented sperm

ICSI attempt	Implantation rate	Birth rate
Conventional	1/24 (4%)	0/24 (0%)
HM	7/21 (33%)	6/21 (29%)

Hazout et al. 2006

Double effect of antioxidants

	Before treatment	After treatment	P-value
DFI (%)	32.4	26.2	0.0004
HDS (%)	17.5	21.5	0.0009

Ménézo et al., RBMOnline 2007

Conclusions - 1

- **Different techniques can be used to assess sperm DNA integrity**
- **Sperm DNA integrity status predicts ART clinical outcome**
- **Cut-off values depend on technical aspects and vary between labs**

Conclusions - 2

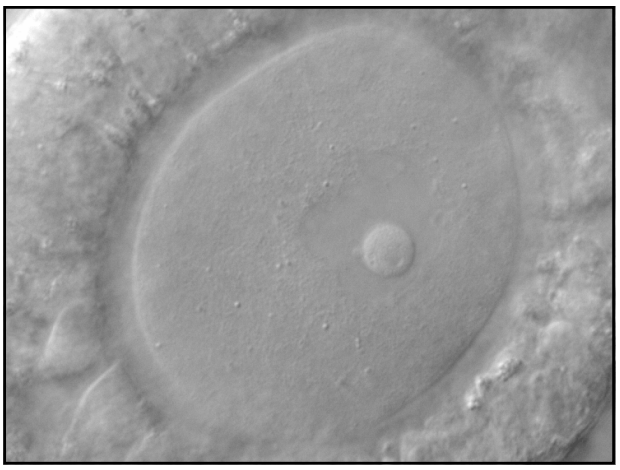
- **Therapeutical solutions now available**
- **Antioxidant treatment**
- **Testicular sperm if antioxidants fail**
- **Potential adverse effect of antioxidants**
- **High-magnification ICSI**
- **Chromatin assays: a guide for prognosis and the choice of adequate treatment**

The oocyte-cumulus dialog and mammalian embryonic development

David F. Albertini, Ph.D.
Hall Professor of Molecular Medicine
Kansas University Medical Center
July 1, 2007

Outline

- Developmental contingencies for oocyte-granulosa communication
- The molecular and cellular components of the germ/soma interface
- Impact of preovulatory communication on developmental competence
- Impact of periovulatory communication on developmental competence



Developmental contingencies for oocyte-granulosa communication

- establish primitive contacts during follicle formation-quiescence
- elaborate junctional contacts at onset of follicle growth-nutritional support
- remodel at acquisition of meiotic competence-impose arrest state
- assure cumulus autonomy during follicular expansion-reinforce phenotypes
- remodel in response to LH surge-meiotic progression

The molecular and cellular components of the germ soma interface

- Intercellular junctions:gap, adhesion, signalling
- Zona Pellucida: more than a landing pad
- Secretion and uptake machinery for exchanged ligands:BMP-15, GDF-9,KL
- Transzonal projections(TZPs): hormonally regulated transfer devices
- Oolemmal mosaic membrane array:multiple inputs, multiple outputs

Impact of preovulatory communication on developmental competence

- control the pace of oocyte and follicular growth
- maintain cumulus phenotypic identity to prevent the onset of atresia
- coordinate the acquisition of meiotic competence with meiotic arrest signals
- impose gradients for the localization of oocyte RNAs and proteins

Factors affecting oocyte/embryo quality

- shortening or prolonging follicular phase
- distortions in ratios of secreted factors from oocyte or granulosa
- receptiveness or overdosing for gonadotropin responsiveness
- removal of COC from the follicle
- removal of the cumulus cells
- the infinite dilution

Consequences of oocyte deficiencies on embryo performance

- growth restriction leads to organelle decrease, misplacement, and loss of function
- precocious resumption of meiosis reduces cytoplasmic maturation
- introduces variations in mRNA and protein stability
- Multiple outcomes:cell cycle timing, ZGA onset, allocation of icm/te, modified checkpoint regulation, loss of synchrony with implantation signals.

Conclusions

- immediate environment of oocyte dictates an organizational plan to carry out pre-implantation development
- disturbances during pre/peri ovulatory period have long term consequences in initial cell cycles of embryo
- mechanism involves maternal factors that interact with new zygotic gene products

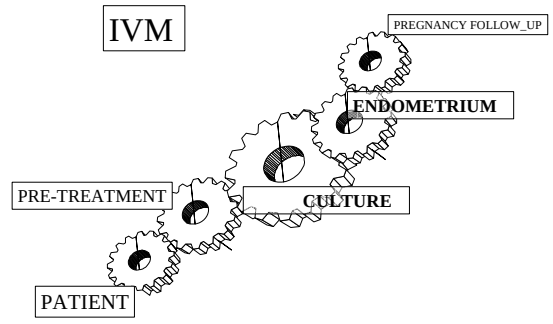
Impact of periovulatory communication on embryonic development

- cumulus signalling "corticalizes" nuclear and cytoplasmic maturation
- remodelling of the zona both facilitates exchange of factors, their availability, and impacts sperm penetration
- critical time window to synchronize cumulus metabolic up-regulation with oocyte

ESHRE PRE-CONGRESS COURSE, Lyon 2007

STRATEGIES to IMPROVE CLINICAL IVM of OOCYTES

Johan Smitz
Follicle Biology Laboratory
Free University Brussels (VUB)



STATUS 2007

- good results : PCO background
- ICSI obligatory
- Not always enough (10) oocytes
- High nr of ET vs conventional IVF
- More miscarriages than in conventional ART

AIMS

- Adapt IVM to ALL patients ?
- IVM as a first line in ‘easy cases’ ?

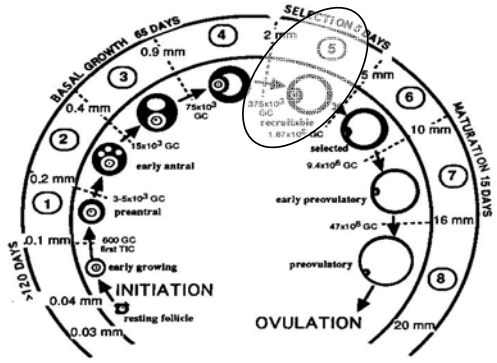
In Vitro Maturation (IVM) : results today

- IMPLANTATION RATE PER EMBRYO TRANSFERRED:

IVM	7 %	→ Factor 2 to 3
IVF/ICSI	14-20 %	

REMEDIES for improved IVM ?

1. **Determine the maturation grade of oocyte - cumulus complexes**
2. **Design appropriate culture environment for variable culture time (few days)**
   - **2.1. Pioneer pharmacology of nuclear arrest of prophase oocytes**
   - **2.2. Investigate effects of substances effecting cytoplasmic maturation**
3. **Investigate relation gene expression - developmental competence → predictive tests**



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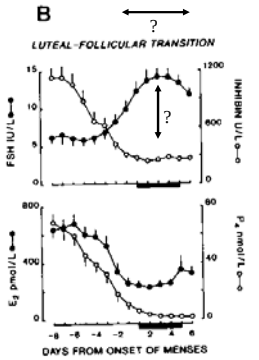

5 June, 2007 8

problems inherent to the ovarian status

- **1 n months '<u>history</u>' of the follicle**
  - **Oocyte quality pre-determined ?**
- **2 <u>asynchrony</u> in the FSH-recruited follicle cohort**
  - **Can we 'treat' it ?**

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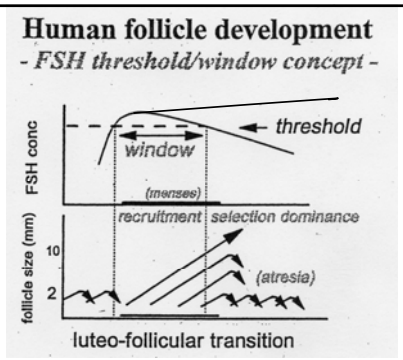
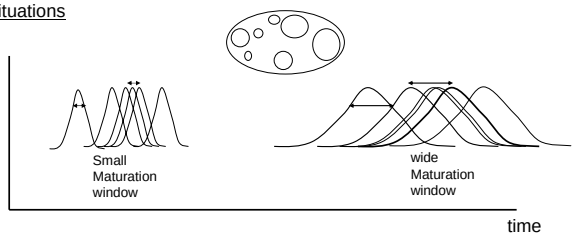


5 June, 2007 Adapted from Fauser (1997) 10

DEALING WITH A COHORT :
maturation capacity window

2 situations



How long does the oocyte in the follicle keep its full potential ?
Window = ? 2-3 days ?

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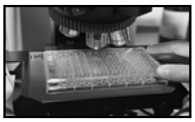
MODELLING

110-130µm

Equal culture conditions



Synchronous development

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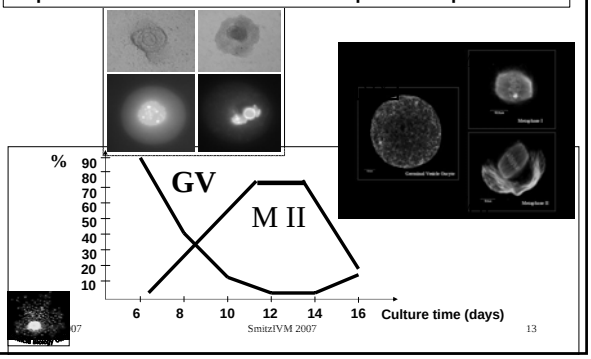
Experience from MOUSE : maturation potential upon LH stim



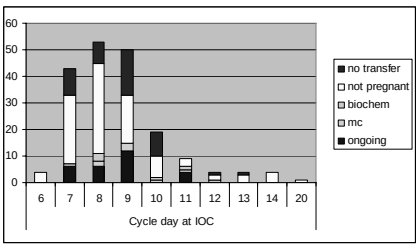
ART REGISTRIES : OUTCOMES

- **PRETREATMENT**

  Desensitisation = + 5% pregnancies

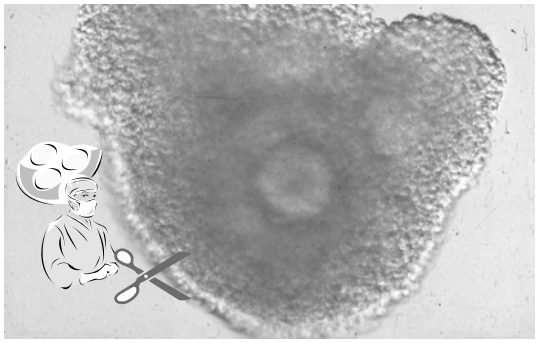
  (throughout different gonadotrophin protocols)

‘Natural’ cycle day at oocyte retrieval

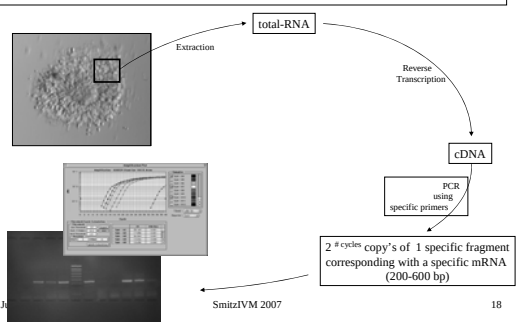


Dr A.M. Suikkari (Vaestolitto, Helsinki)

PROBLEM 1 : HETEROGENEITY OF THE COC



RT-PCR technology



POTENTIAL MARKERS in cumulus for Follicle dev stage

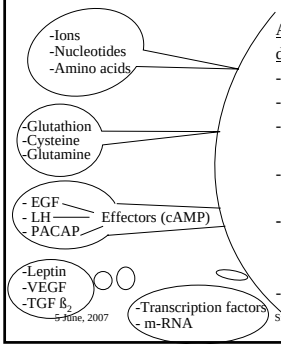
Expression of:

- LH-receptor expression
- AMH
- Mucification enabling factor(s)
- Steroidogenic enzymes: aromatase
- growth factors / GF receptors

PROBLEM 2 :

Cumulus cells are removed
or drop off during prolonged culture

COMMUNICATION GC - OOCYTE



Absence of communication →
defects on fertilisation

- premature migration of cortical granules
- decrease in penetrability of sperm
- decrease in acrosome reaction

Syngamy

- inability to form the male pronucleus

Development

- decreased supplies of transcripts to be activated and translated during early pre- and post implantation development
- inadequate embryonic genome activation

PROBLEM 3

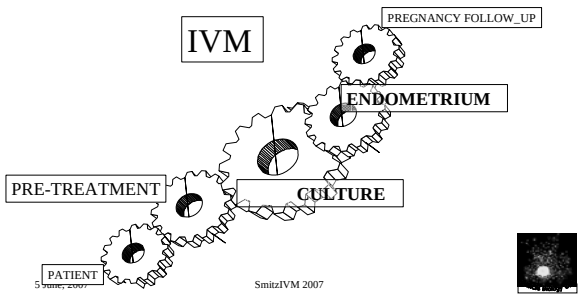
nuclear maturation progresses inevitably,
but within variable time frames :

When to do ICSI ?

CYTOPLASMIC MATURATION

- **progresses in parallel and partly independent from nuclear maturation**
- **essential contribution from metabolic coupling with the cumulus cells (glutathion, effects from growth factors)**
- **during oocyte maturation (few hours) :**
  - **translation of stockpiled dormant transcripts**
  - **changes in protein neosynthesis profile**
  - **modification in adenylation of certain mRNAs**
- **some proteins have been characterised : essential for cell cycle control (c-mos, cyclin B)**

Attempts to improve



Attempts to improve IVM

- Patient selection
- Stimulate ovary or natural cycle ?
- Culture conditions
- Clinical results
- Future aims

Oocyte In Vitro Maturation

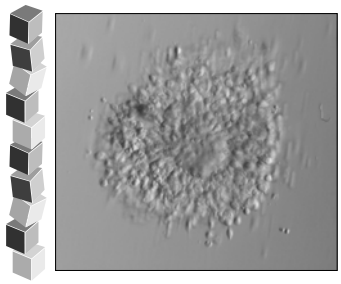
- Clinical management: SOPs have been developed
  - patient pretreatment: stimulation FSH/HCG
  - retrieval technique of OCC
  - endometrial preparation
- Laboratory techniques: to be improved
  - maturation technique(s)
  - maturation medium
  - fertilisation technique
  - safety

CYTOPLASMIC MATURATION

- **depends on culture conditions**

medium composition
cellular environment

IN VITRO MATURATION



SMALL ANTRAL FOLLICLE.

↓

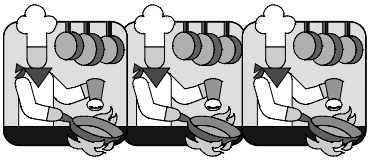
OOCYTE - CORONA - CUMULUS

↓

HOW TO CULTURE ?

Measure mRNA in cumulus biopsy

IVM
1992- 2007



FUTURE

DEVELOPMENTAL COMPETENCE

- **oocytes acquire the potential for developmental competence in the period preceding the maturation stimulus**
- **a prolonged COC culture would enable the oocyte to complete biochemical processes essential for further embryonic development**

  - transcribe mRNAs
  - translate essential proteins
  - and / or posttranslationally modify them

<u>ESSENTIAL</u> for further embryonic development

Stop **Start**

BRAKE ON NUCLEAR PROGRESSION

24 → 48 → 72 ... HOURS

retrieve oocyte cumulus → space for intervention on oocyte → **resume meiosis** → fertilise → culture

continued dialogue with cumulus

add activators of cytoplasmic maturation

non invasive analyses

Decision level

- energy metabolism
- stores of mRNA
- increase glutathione

PROOF OF CONCEPT & "pre-clinical" safety

MOUSE IVM MODEL

1. relation follicle immaturity – oocyte competence
   Immature follicle < OCC → IVF + ET → Poor results.

2. helping effects of cumulus in culture

   immature follicle < oocytes → cumulus-surrounded
   immature follicle < oocytes → cumulus-free

3. Pre-incubation (nuclear arrest) improves oocytes' competencies

   immature follicle

   → < cumulus-surrounded oocytes → preincubation 24 hrs → maturation → IVF ET
   → < cumulus-surrounded oocytes → immediate maturation → IVF ET

Immature OCC: culture issues to develop in HUMAN

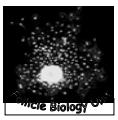
Cytoplasmic immaturity improvement

HYPOTHESIS

→ Need for prolonged pre-maturation phase

Determine TIMING for oocyte fertilisation

Preliminary safety analysis



Stop **Start**

BRAKE ON NUCLEAR PROGRESSION

24 → 48 → 72 ... HOURS

& Reversal of arrest

Proof of concept study :

n= 66 patients

170 OCC obtained pré-HCG and 36h post-HCG

Oocytes can remain 'nuclear'arrested in-vitro for 72 hours

Reversal of arrest (72h + 24-30h): 68 % resumed meiosis

Global efficiency (PB's /retrieved OCC) : 50 %

5 34

Stop **Start**

BRAKE ON NUCLEAR PROGRESSION

24 → 48 → 72 ... HOURS

& Reversal of arrest

Proof of concept & Safety study :

n= 84 patients

250 OCC obtained 36h post-HCG

Oocytes remain 'nuclear'-arrested in-vitro for 48 h

5 June, 2007 SmitzIVM 2007 35

ACKNOWLEDGEMENTS

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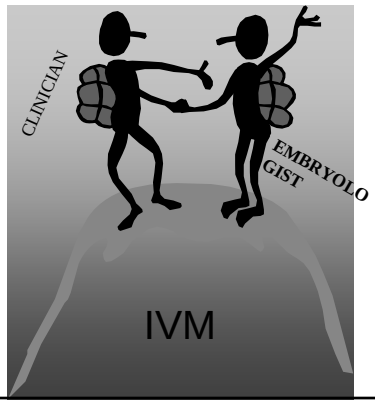
- Peter Platteau & Carola Albano
- Free University Brussels
- Novo Nordisk

5 June, 2007 SmitzIVM 2007

Conclusions

- Perfection of IVM could lead to increased embryo yields
  (savings for social security / less invasive for patient)
- Improvements in IVM culture to be expected from increased knowledge
  - » Tools from molec biol
  - » Measure cytoplasmic maturation
- Standardisation of IVM procedure is needed
- Control of nuclear arrest could improve IVM technology



ESHRE Joint SIG Embryology and SIG Andrology
Pre-Congress Course:
"To what extent do male and female gametes influence embryo development?"

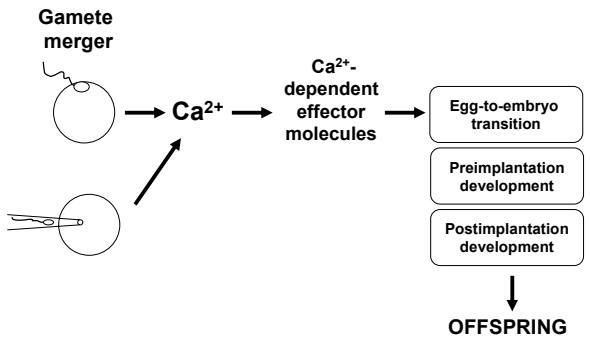
Sperm-egg fusion, ICSI and egg activation: *what's new?*

Janice P. Evans, Ph.D.
Johns Hopkins University
Bloomberg School of Public Health
Baltimore, MD, USA

Lecture Overview and Learning Objectives

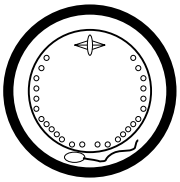
- Review of the mechanisms underlying egg activation
- Comparison of egg activation responses in conventional IVF eggs and ICSI-fertilized eggs
  - Case study: Abnormalities in an egg activation response (membrane block establishment) in ICSI-fertilized eggs

From fertilization to offspring



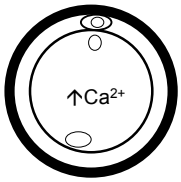
Changes occurring upon fertilization

Unfertilized



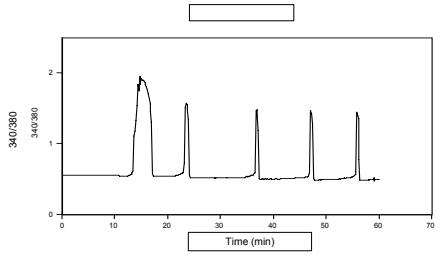
- Arrested at metaphase II
- Cortical granules in cortex

Fertilized



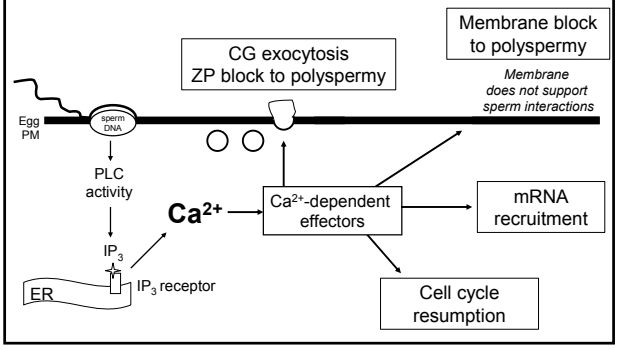
- Cell cycle resumption
  - Exit from metaphase II arrest
  - Progression to mitosis
- CG exocytosis
  - ZP block to polyspermy
- Membrane block to polyspermy

Oscillatory Ca^{2+} rises in mammalian eggs



Gardner, Williams and Evans
Reproduction (2007) 133, 383-393

Intracellular calcium signaling and egg activation responses



Post-fertilization changes in eggs:
Candidate Ca^{2+}-dependent effector molecules for different egg activation events

- Ca^{2+}-Calmodulin (CaM)-dependent kinase (**CaMKII**)
  - Exit from metaphase II arrest
  - Cortical granule exocytosis
- Myosin light chain kinase (**MLCK**)
  - Polar body emission (meiotic cytokinesis)
  - Cortical granule exocytosis
- Protein Kinase C (**PKC**)
  - Cortical granule exocytosis

Post-fertilization changes in eggs:
Different relative Ca^{2+} dependence for different egg activation events

- *From studies in mouse with experimentally manipulated $[Ca^{2+}]_{cyt}$ increases (Dev. Biol. 250:280-291):*
  - minimal Ca^{2+} input - cortical granule exocytosis
  - moderate Ca^{2+} input - metaphase II exit
  - higher Ca^{2+} input - progression to embryonic interphase (pronuclear stage), mRNA recruitment

Post-fertilization changes in eggs

- **Cortical granule exocytosis**
  - Initiation requires 1-4 Ca^{2+} rises
  - Several Ca^{2+}-dependent effectors likely: CaMKII, MLCK, PKC, others
- **Cell cycle resumption**
  - Metaphase II exit triggered by 4-8 Ca^{2+} rises
  - CaMKII activity leads to activation of the Anaphase-Promoting Complex, leading to cyclin B degradation

Post-fertilization changes

- **Recruitment of mRNAs for translation**
  - Progressively more with 8 to 24 Ca^{2+} rises
  - Required for initiation of transcription in one-cell mouse embryos
- **Membrane block to polyspermy**
  - Ca^{2+} regulates timing of establishment
  - CaMKII participates, but not sufficient
  - Supplemental trigger linked with some event(s) associated with sperm-egg interaction

Differences in Ca^{2+} oscillations in ICSI-fertilized eggs

- **Complete failures**
  - Especially in certain pecies (equine, bovine)
- **Temporal**
  - Delayed initiation
  - Premature cessation
- **Spatial**
  - Differs from distinct point of origin at sperm entry point with conventional IVF
- *Varies with how sperm is prepared for injection*

Differences in activities of Ca^{2+} - dependent effector molecules in ICSI-fertilized eggs

- *Very little known!*
  - One analysis of CaMKII activity in mouse eggs post-IVF or post-ICSI at two post-insemination time points (*Mol. Human Reprod.* 2007, 13, 265-272)
    - First Ca^{2+} rise
    - Rise occurring with second polar body emission
  - CaMKII activities similar in IVF- and ICSI-generated zygotes (although functional differences still possible)

Case study in egg activation:
The membrane block to polyspermy

- Post-fertilization change in egg membrane function
  - Pre-fertilization - receptive to sperm
  - Post-fertilization - unreceptive to sperm
- Functions in conjunction with the ZP block to polyspermy
- Occurs in most, if not all, species (addressed ahead)

Polyspermy and triploid embryos in mammals

- Triploidy detected in ~1% of conceptuses
  - 10% of spontaneous abortions
  - 30% of chromosomally abnormal spontaneous abortions
- Majority of triploid conceptuses are the result of fertilization by two sperm
- Incidence of polyspermy in mammals is 1-2%
  - 3-30% with delayed mating/insemination

Summarized in Gardner and Evans
Reprod. Fertil. Dev. 18, 53-61

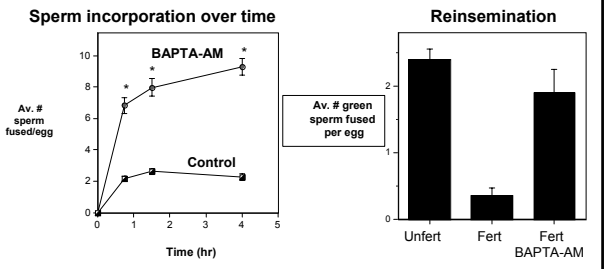
Perivitelline sperm

PVS sperm	Species	Interpretation
10s-100s	Rabbit, pika, pocket gopher, mole	• Membrane block highly effective • ZP block relatively ineffective
1-10	Mouse, human, rat, guinea pig, cat, ferret, pig, cattle	• Utilization of both ZP and membrane blocks
Rare	Dog, sheep, field vole	• ZP block highly effective • Inconclusive regarding membrane block

Yanagimachi, 1994
Florman and Ducibella, 2006
Gardner and Evans, 2006

Impaired membrane block establishment in ZP-free eggs with no sperm-induced increase in $[Ca^{2+}]_{cyt}$

(10 μM intracellular Ca^{2+} chelator, BAPTA-AM)



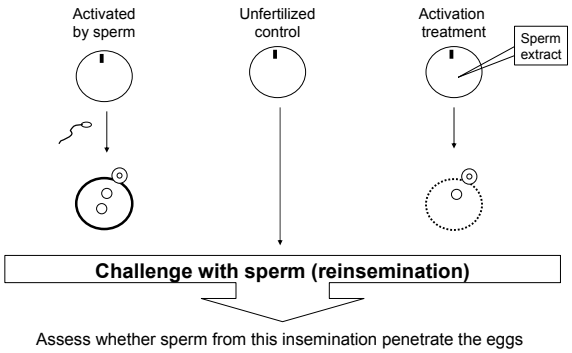
McAvey, Wortzman, Williams, Evans
Biol. Reprod., 67:1342-1352

Gardner, Williams and Evans
Reproduction (2007) 133:383-393

Failures in the membrane block

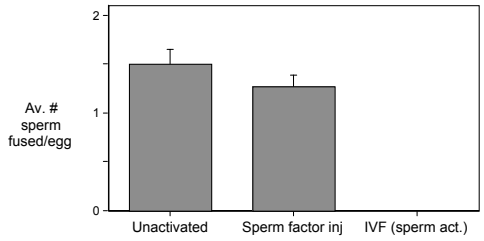
- Parthenogenetically-activated eggs
  - *Dev. Biol.* 71:22; *Mol. Reprod. Dev.* 34:65 and 52:183
- ICSI-fertilized eggs
  - *Mol. Reprod. Dev*. 44:256 and 52:183
- Both parthenogenesis and ICSI trigger Ca^{2+} rises that differ from those with fertilization.
- Hypothesis: Only "fertilization-like" Ca^{2+} rises can trigger membrane block establishment.

Egg activation by sperm versus sperm extract-induced parthenogenesis



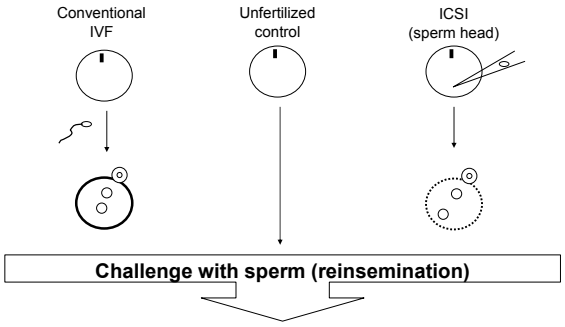
Assess whether sperm from this insemination penetrate the eggs

Activation of eggs with sperm factor does <u>not</u> induce membrane block establishment



Wortzman-Show, Kurokawa, Fissore and Evans
Mol. Human Reprod. in press

Egg activation by conventional IVF versus ICSI



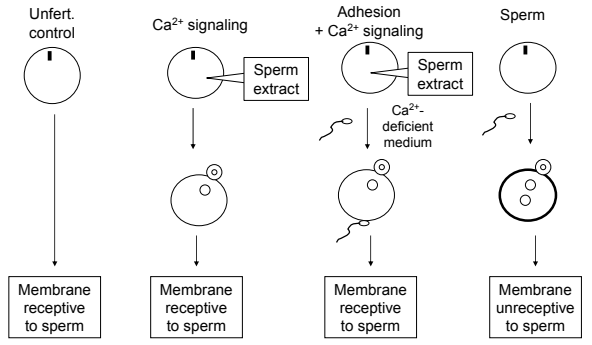
Assess whether sperm from this insemination penetrate the eggs

Variations on ICSI do <u>not</u> induce membrane block establishment

- Sperm head
- Whole sperm (to include the tail)
- Permeabilized sperm (to facilitate factor release in the egg)
- Multiple sperm heads (to increase dose of sperm factors)

Wortzman-Show, Kurokawa, Fissore and Evans
Mol. Human Reprod. in press

Testing the role of sperm-egg adhesion

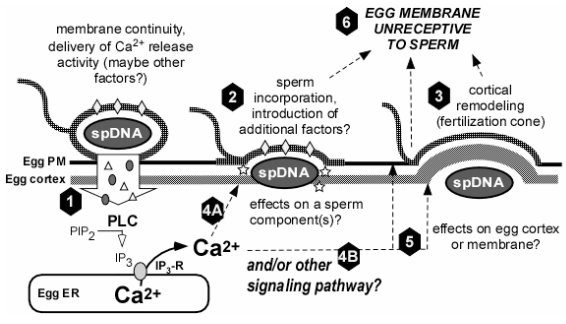


Wortzman-Show, Kurokawa, Fissore and Evans
Mol. Human Reprod. in press

The membrane block and sperm-egg interactions

- Many papers on gamete membrane interactions use "*sperm-egg fusion*" to refer collectively to all events leading up to fusion (e.g., sperm-egg binding).
- In considering ICSI and the membrane block, we also need to broaden "sperm-egg fusion" to *events linked with fusion.*
  - The actual cellular event of membrane fusion itself is unlikely to be sufficiently catalytic to trigger a change throughout the egg.
  - Adds very little new membrane (a mouse sperm head is only ~0.14% of the surface area of a mouse egg) - unlikely to be a dilution effect.

Membrane block establishment and events linked with sperm-egg fusion



Establishment of the membrane block to polyspermy involves both gametes

- Sperm delivers signal(s) that trigger membrane block establishment
  - Sperm-induced increase in intracellular calcium
  - Some event(s) associated with sperm-egg membrane interactions
- Egg responsiveness to the signal
  - Post-ovulatory aged eggs are less able to establish the membrane block to polyspermy (*Mol. Human Reprod*. 2005 11:1-9).

Final thoughts

- ICSI leads to live births, although ICSI does not faithfully mimic all events of the egg-to-embryo transition
  - Differences in Ca^{2+} rises
  - Membrane block establishment
    - inconsequential in infertility clinics (no risk of polyspermy for ICSI-fertilized eggs)
- *Are other events of egg activation and early embryogenesis modified in ICSI-fertilized eggs?*
  - *Advantages to having gamete merger occur through sperm-egg interactions?*
- *Are other events of egg activation and early embryogenesis sufficiently similar to conventional IVF embryos?*

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- Allison Gardner

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- Jason Knott
- Keith Jones

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Selected references - Review articles

Ducibella T, Schultz RM and Ozil JP (2006) Role of calcium signals in early development. *Semin Cell Dev Biol*. 17, 324-32.

Malcuit C, Kurokawa M and Fissore RA (2006) Calcium oscillations and mammalian egg activation. *J. Cell. Physiol*. 206, 565-73.

Selected references - ICSI and Ca^{2+}

Bedford SJ, Kurokawa M, Hinrichs K and Fissore RA (2003). Intraceullar calcium oscillations and activation in house eggs injected with stallion sperm extrats or spermatozoa. *Reproduction* 126,489-499.

Kurokawa M and Fissore RA (2003) ICSI-generated mouse zygotes exhibit altered calcium oscillations, inositol 1,4,5-trisphosphate receptor-1 down-regulation, and embryo development. *Mol. Human Reprod*. 9, 523-33.

Malcuit C, Maserati M, Takahashi Y, Page R and Fissore RA (2006) Intracytoplasmic sperm injection in the bovine induces abnormal $[Ca^{2+}]_i$ responses and oocyte activation. *Reprod. Fertil. Dev*. 18, 39-51.

Nakano Y, Shirakawa H, Mitsuhashi N, Kuwabara Y and Miyazaki S (1997) Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Mol. Human Reprod*. 3, 1087-1093.

Tesarik J, Sousa M and Testart J (1994) Human oocyte activation after intracytoplasmic sperm injection. *Human Reprod*. 9, 511-518.

Selected references - Membrane block to polyspermy

Gardner AJ and Evans JP (2006) Mammalian membrane block to polyspermy: new insights into how mammalian eggs prevent fertilisation by multiple sperm. *Reprod. Fertil. Dev*. 18, 53-61.

Gardner AJ, Williams CJ and Evans JP (2007) Establishment of the mammalian membrane block to polyspermy: Evidence for calcium-dependent and -independent regulation. *Reproduction* 133, 383-393.

Gardner AJ, Knott JG, Jones KT and Evans JP (2007) CaMKII can participate in but is not sufficient for the establishment of the membrane block to polyspermy in mouse eggs. *J. Cell. Physiol*. 212, 275-280.

Wortzman-Show GB, Kurokawa M, Fissore RA and Evans JP (in press) Calcium and sperm components in the establishment of the membrane block to polyspermy: studies of intracytoplasmic sperm injection (ICSI) and activation with sperm factor. *Mol. Human Reprod*.

Optimizing In-Vitro Fertilization Systems

Dr David Mortimer, PhD
Oozoa Biomedical Inc
Vancouver, BC, Canada

Learning Objectives

1. **To identify the biological, chemical and physical factors that influence the design and organization of in-vitro fertilization systems in the human ART laboratory.**
2. **To recognize extrinsic factors that can impact adversely upon human fertilization *in vitro*.**
3. **To apply process analysis to optimize in-vitro fertilization systems in the human ART laboratory.**

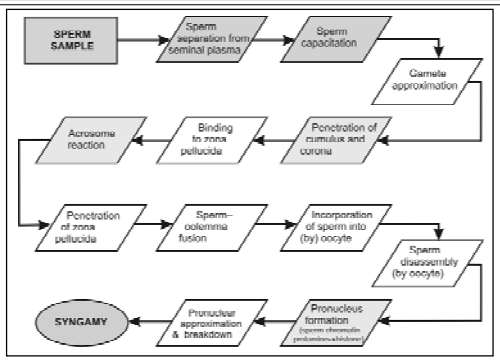
In-Vitro Fertilization

Fertilization: **The fertilization of a mature oocyte to produce a normal zygote that is capable of developing into a normal, competent embryo.**

IVF Process: **The ability of the sperm preparation and culture systems to support sperm capacitation ands the fertilization of mature oocytes.**

Confounders:
- **Sperm dysfunction**
- **Sperm DNA damage (inherent / iatrogenic)**
- **Anti-sperm antibodies**
- **Incomplete / abnormal ooplasmic maturation**

The Process Of Fertilization



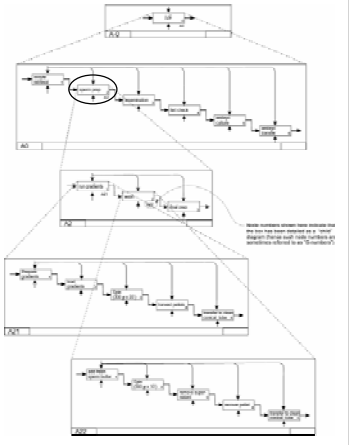
Process Analysis: *Basic Principles*

What is a process? Inputs | "Something happens" | Output

IVF is a Process: Sperm + Oocytes | "Lab systems" | Embryos
but it can't be analyzed as such because "Lab Systems" includes too many "lower level" Process.

Process mapping:

- **Draw out the system and define each component Process.**
- **Dig deep: each Process must have no subordinate or derivative (sub)process(es).**
- **Identify and define the all of the factors that control each Process.**



Process Mapping

IDEF0 process mapping of IVF systems

Mortimer & Mortimer : *Quality and Risk Management in the IVF Laboratory, CUP 2005*: Fig 7.5

Process Control for In-Vitro Fertilization

Indicators: • Define the process to be monitored
• Must measure only the intended process
• Must minimize extraneous effects

Indicators for IVF fertilization:

Fertilization rate	**% oocytes inseminated with ≥2PN**
Normal fertilization rate	**% oocytes inseminated with 2PN**
Polyspermy rate	**% oocytes inseminated with >2PN**
Low or failed fert'n rate	**% of cases with <25% or 0% zygotes**

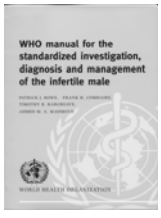
Indicators for ICSI fertilization:

Fertilization rate	**% oocytes injected with 2PN**

Why Optimize Fertilization *In Vitro*?

- **Reduce the risk of poor or failed fertilization:**
  - **Select the most functional sperm**
  - **Provide optimum support for sperm capacitation and fertilization**
  - **Avoid iatrogenic sperm dysfunction**
- **Maximize the number of embryos:**
  - **Maximize the fertilization of the available MII oocytes**
- **Optimize embryo quality:**
  - **Select sperm with the best DNA**
  - **Prevent sperm DNA damage during processing**
- **Reduce the unnecessary use of ICSI:**
  - **Maximize the number of zygotes produced**
  - **Prevent acrosomal enzymes damaging the oocyte**
    (e.g. Morozumi & Yanagimachi, PNAS 102: 14209-14, 2005)

The WHO and Structured Management



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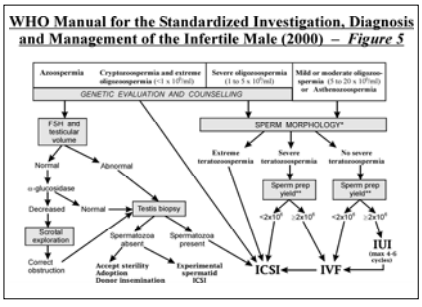


Sperm Surface ASABs and IVF

Royal Women's Hospital (Melbourne) IVF Unit

%IgG	%IgA	# men	Oocytes fertilized (%)	
≥80%	≥80%	8	18/66	27%
+ve	<80%	9	47/65	72%
–ve	–ve			75–80%

Clarke *et al.* (1985) Am. J. Reprod. Immunol. Microbiol., 8: 62.

ASABs and Management

BINDING & SITE*	MANAGEMENT
Tail-tip only	Not clinically significant
<50% head / midpiece / tail	Not clinically significant
50–80% midpiece / tail	Try IUI? or go to IVF
50–80% head	IVF (adjust sperm numbers?)
>80% midpiece / tail	Try IVF or go to ICSI
>80% head	ICSI

*e.g. by Immunobead test

SOURCE: Rowe *et al.* (2000) *WHO Clinical Manual for the Standardized Investigation, Diagnosis and Management of the Infertile Male.* Cambridge University Press, Cambridge, UK.

An Argument Against "ICSI For All"

What happens if you use ICSI on someone for whom IVF would work?

Parameter	*Criteria*	IVF Rate	IVF #	ICSI Rate	ICSI #	*Criteria*
COCs retrieved			11		11	
Maturity	*% MIIs*	(85%)		85%	9.35	
ICSI damage rate				-3%	9.07	
Fertilization rate	*as % of COCs inseminated*	80%	8.80	70%	6.35	*of MIIs injected*
Cleavage rate	*% of zygotes*	98%	8.62	98%	6.22	
Utilization rate	*Day 3 embryos for ET or cryo*	65%	5.61	65%	4.04	
Embryos transferred		2		2		
	Implantation rate (fresh)	30%	60%	30%	60%	Equal chance
Embryos cryopreserved			3.61		2.04	
Cryosurvival rate		75%	2.70	75%	1.53	
	Implantation rate (frozen)	20%	54%	20%	31%	
Cumulative implantation potential			114%		91%	
Loss of outcome for patient				23%		

Sperm Functional Assessment or "SFA"

- **Semen Analysis**
  - **Comprehensive as per WHO'99 and ESHRE/NAFA**
  - **Detailed morphology including TZI**
- **Trial Wash**
  - **PureSperm gradient: determine quantitative & qualitative yields**
- **Anti-Sperm Antibodies**
  - **Direct IBT with "GAM" bead, + isotypes if >20%**
- **Computer-Aided Sperm Analysis (IVOS v12)**
  - **Mucus penetrating population in semen**
  - **Hyperactivation: "HAmax" assay (includes spontaneous control)**

The SFA and ICSI Usage

Sydney IVF (Australia):

- **>90% male factor by WHO criteria**
- **~35% ICSI / ~65% IVF with <5% failed fertilization**

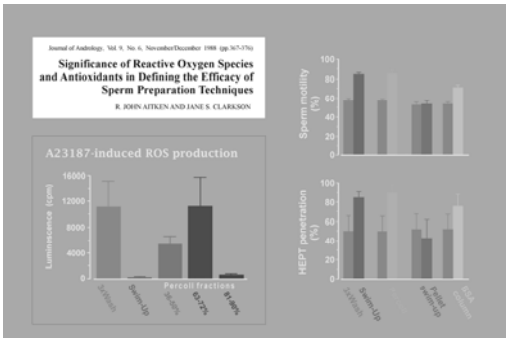
SOURCE: Dr D. Mortimer (unpublished data)

Genesis Fertility Centre (Canada):

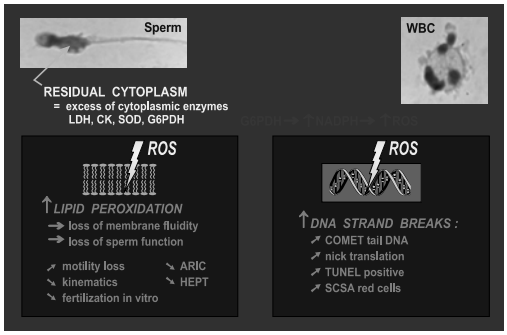
Period	stims/yr	IUI/yr	ICSI	IVF	R/ICSI
Pre-STM	550–600	minimal	60%	40%	active programme
STM	650–700	~600	40%	60%	almost none

SOURCE: Dr S.T. Mortimer (personal communication)

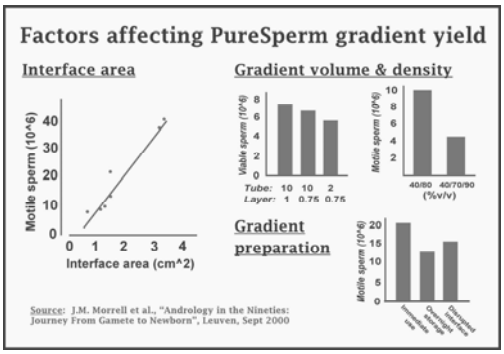
Sperm Preparation and ROS



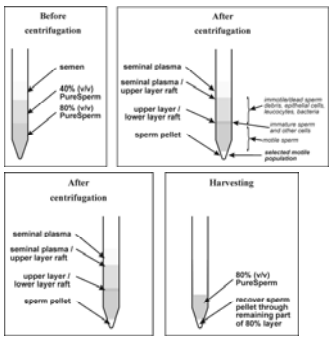
Sperm Preparation : *Dangers of ROS*



PureSperm® Density Gradients



Sperm Preparation: *PureSperm® method*



Standardized method:

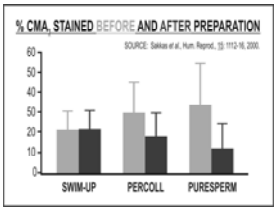
1.5 ml layers / ≤1.5 ml semen

300 ***g*** x 20 min then 500 ***g*** x 10 min

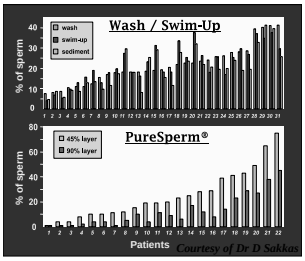
Yield:

- The most dense sperm: these have fully condensed nuclei and no excess cytoplasm.
- Higher proportion of normal sperm.
- Typically >95% progressively motile – if not then it indicates either poor technique or is diagnostic:
  - <85% consider ICSI
  - <80% definitely ICSI

Sperm Preparation & DNA Quality



CMA_3 staining shows decreased presence of protamine (i.e. poorly condensed chromatin).



Endogenous DNA nicks

Sperm Preparation: Conclusions

- **Do not centrifuge seminal spermatozoa:**

  It pellets the good spermatozoa along with abnormal, moribund & dead spermatozoa + other cells, debris, etc, with the risk of ROS generation in the pellet, causing impaired fertilizing ability (damage can be so severe as to prevent fertilization).

  Avoid iatrogenic sperm dysfunction.

Do NOT use the "classical" swim-out from a washed pellet!

- **Use an established, safe, alternative method**
- **Most widely applicable = 2-layer density gradient + 1 wash**

Origins of IVF Media

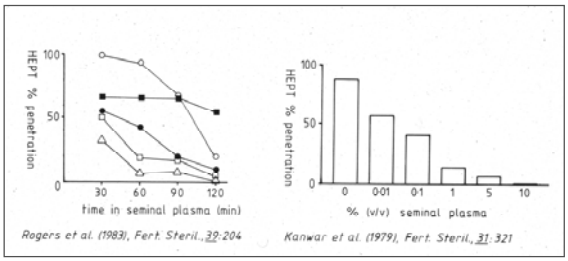
- **Media for somatic cells: e.g. EBSS, MEM, Tyrode's, TALP**
- **Media based on oviduct fluid:**
  - **Tervit's "SOF" (1972)**
  - **Ménézo's "B2" (1976)**
  - **Quinn's "HTF" (1985) | "Advantage" sequential media system**
  - **Mortimer's "STF" (1986) | "M91" | Cook SIVF sequential media**
- ***De novo* formulations: e.g. BWW, Bavister's HECM, Biggers' KSOM**
- **Research animal embryo culture media: e.g. CZB**
- **Most human embryo culture medium development has been based on in-vivo produced mouse zygotes (i.e. not even IVF-derived mouse zygotes)….**

Culture Conditions for Capacitation

Sperm capacitation *in vitro* requires:

- **Separation from seminal plasma (decapacitation factor)**
- **A "capacitating" culture medium:**
  - **Physiological balanced salts to be isotonic and support general homeostasis**
  - **Glucose, usually ~5 mM (range 2.8–6.7 mM)**
  - **Bicarbonate ions, usually 25 mM**
  - **Calcium ions (range 1.7–3.0 mEq/l)**
  - **Albumin as a sterol acceptor: <u>minimum</u> of 10 mg/ml**
    (mid-cycle oviduct fluid contains about 30 mg/ml, serum ~45 mg/ml)

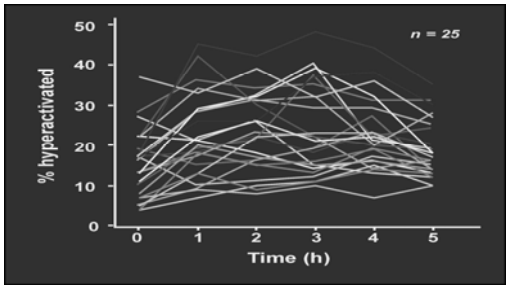
Seminal Plasma and Fertilizing Ability



Rogers et al. (1983), Fert. Steril., 39:204 — Kanwar et al. (1979), Fert. Steril., 31:321

Capacitation Dynamics (1/3)

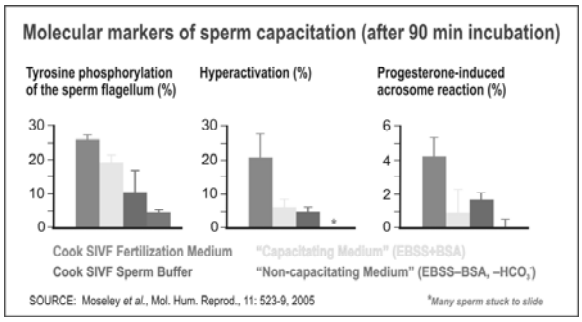
Hyperactivation dynamics vary greatly between men:



<14% HA indicative of need for high sperm numbers at IVF

Karande *et al.* (1990) J. Androl., 11 (sup): P-28 (Abstract 31).

Capacitation Dynamics (2/3)



Sperm Senescence

- **All sperm have a finite lifespan: varies between men and within an ejaculate (also normal *vs* abnormal sperm).**
- **Prolonged exposure to seminal plasma is deleterious.**
- **Sperm have a high metabolic rate under capacitating conditions: leads to ROS generation / "burn-out".**
- **Once capacitated, sperm are highly labile: spontaneous and induced acrosome reactions.**
- **Acrosome-reacted sperm cannot fertilize.**
- **Sperm die soon after the acrosome reaction *in vitro* / *in vivo*.**
- **Therefore: collect after OPU and process immediately**
  use a non-capacitating "holding" medium, &
  keep at room temperature to slow metabolism.

Conclusions: General Principles

THINK LIKE A SPERM!

- **The <u>biology</u> controls *everything* that is done in the IVF Lab.**
- **The chemistry and physics must provide optimum support to the biology.**
- **The engineering must reflect the physical and chemical requirements.**
- **The design and organization of the lab systems must reflect all of the above.**

Learning Objectives

1. **To identify the biological, chemical and physical factors that influence the design and organization of in-vitro fertilization systems in the human ART laboratory.**
2. **To recognize extrinsic factors that can impact adversely upon human fertilization *in vitro*.**
3. **To apply process analysis to optimize in-vitro fertilization systems in the human ART laboratory.**

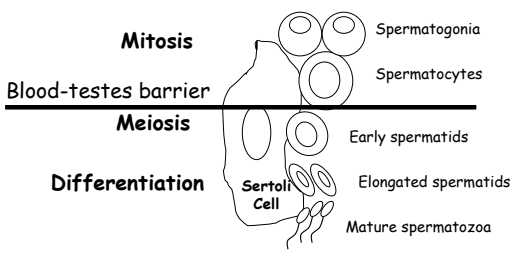
Course 2 - Joint SIG Embryology and SIG Andrology
"To what extent do male and female gametes influence embryo development?"

Selecting sperm: by Nature and in the Laboratory

Denny Sakkas

Department of Obstetrics, Gynecology and Reproductive Sciences
Yale School of Medicine
Yale University
USA

Spermatogenesis



In a process that produces tens of millions of spermatozoa a day: How does nature police spermatogenesis to provide the best spermatozoa and how can these fail?

Two systems that may police abnormal sperm production:

Apoptosis

DNA repair and remodeling

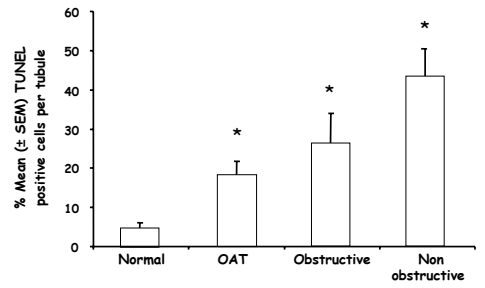
Apoptosis During Spermatogenesis

- **In addition to proliferation and differentiation the outcome of spermatogenesis is affected by the extent of programmed cell death (apoptosis).**
- **Apoptosis occurs normally and continuously in the testis throughout life.**

Apoptosis has two possible roles during normal spermatogenesis:

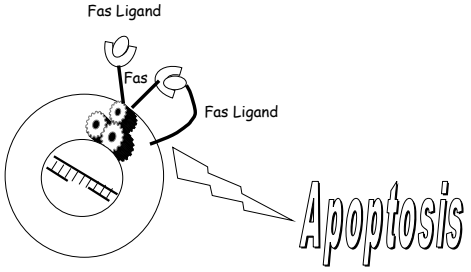
1. Limitation of the germ cell population to numbers that can be supported by the Sertoli cells
2. Selective depletion of abnormal sperm cells enabling the production of normal mature spermatozoa

TUNEL Staining in Testis From Men With Azoospermia and OAT Compared to Normal Controls

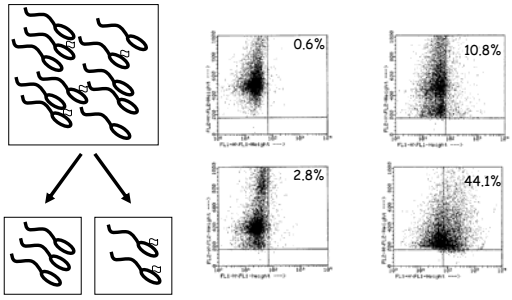


When challenged with abnormal sperm production how effective is apoptosis in providing normal ejaculated spermatozoa?

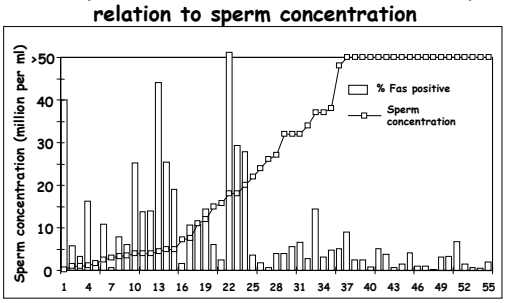
APOPTOSIS
-programmed cell death



Flow cytometry analysis of ejaculated sperm from samples exhibiting low and high Fas positivity

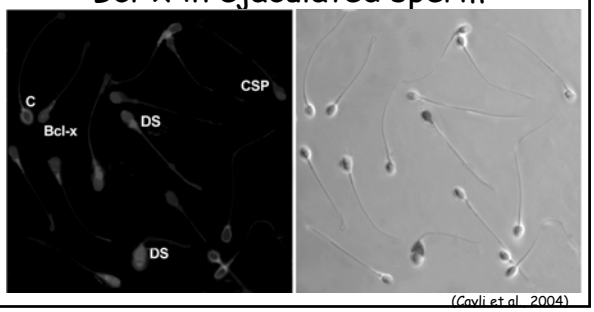


The percentage of ejaculated human spermatozoa labeled positive with anti-human Fas antibody in relation to sperm concentration



(Sakkas et al., 1999)

Double staining of caspase-3 and Bcl-x in ejaculated sperm



(Cayli et al. 2004)

Double staining of ejaculated spermatozoa

- Shorter tail length
- Larger head size

Implies that spermatogenesis has proceeded abnormally

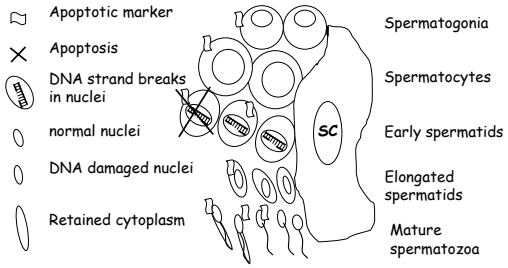
(Cayli et al. 2004)

Presence of apoptotic marker proteins in ejaculated human spermatozoa

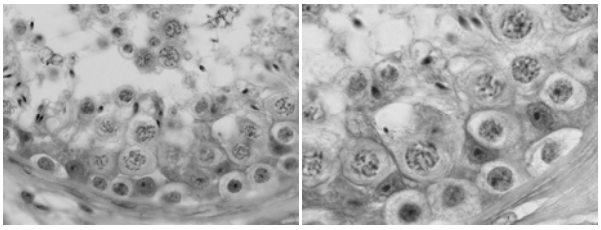
For example: Bcl-x, p53, caspases and Annexin V

Sakkas et al. 1999 and 2002
Barroso G et al. 2000
Oehninger et al. 2003
Cayli et al. 2004
Grunewald et al 2005

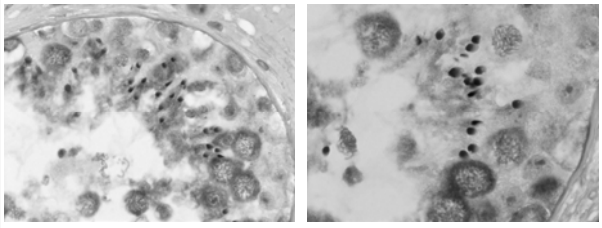
A hypothetical model of apoptosis during abnormal spermatogenesis



Caspase 9 expression in human testes is largely localized in the Sertoli cells



Cleaved Parp-1 expression in human testes



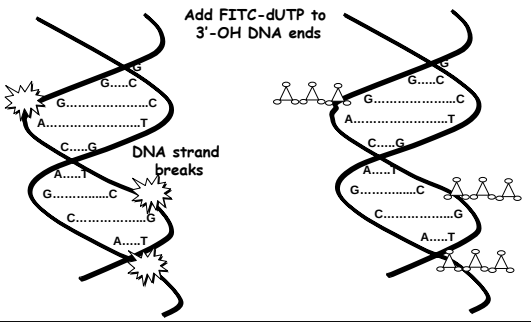
DURING SPERMATOGENESIS AN "ABORTIVE APOPTOSIS" MAY OCCUR LEADING TO THE PRESENCE OF ABNORMAL SPERMATOZOA IN THE EJACULATE

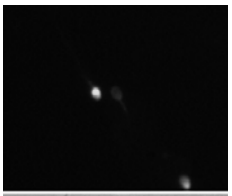
Two systems that may police abnormal sperm production:

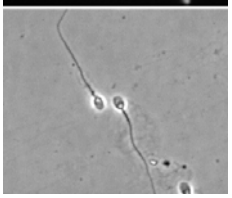
Apoptosis

DNA repair and chromatin remodeling

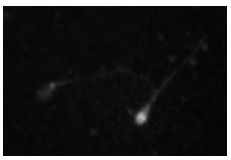
DNA Strand Breaks



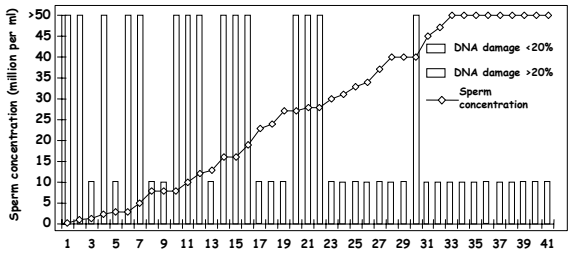


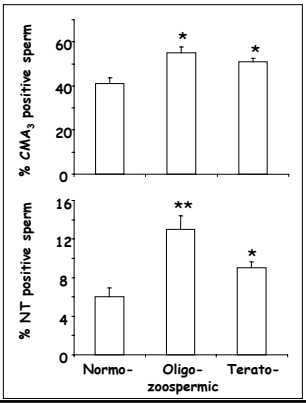


Assessment of endogenous nuclear DNA damage in ejaculated human spermatozoa using endogenous in situ nick translation or TUNEL



The percentage of ejaculated human spermatozoa per sample containing DNA damaged nuclei in relation to sperm concentration



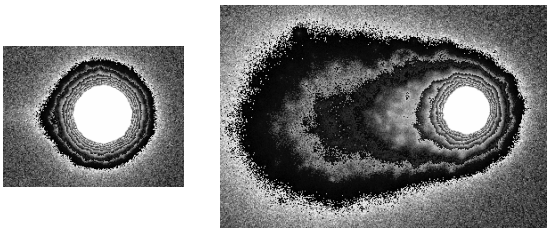


Chromomycin A3 (CMA3) indirectly demonstrates a decreased presence of protamines.

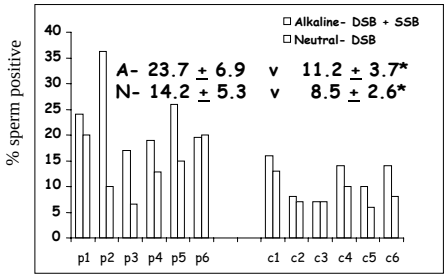
In situ nick translation (NT) demonstrates the presence of DNA strand breaks.

(Tomlinson et al., 2001)

COMET ASSAY OF SPERMATOZOA SHOWS BOTH SINGLE AND DOUBLE DNA STRAND BREAKS



Percentage of ejaculated sperm positive for DNA strand breaks after analysis by the Comet assay in either alkaline or neutral conditions



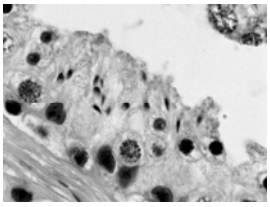
(Sakkas et al., 2002)

DNA Repair

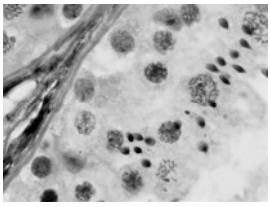
Base excision repair (BER) proteins

excise and replace damaged DNA bases, mainly those arising from endogenous oxidative and hydrolytic decay of DNA

Expression of the DNA repair proteins poly(ADP-ribose) polymerase (PARP1) and apurinic/apyrimidinic endonuclease (APE)

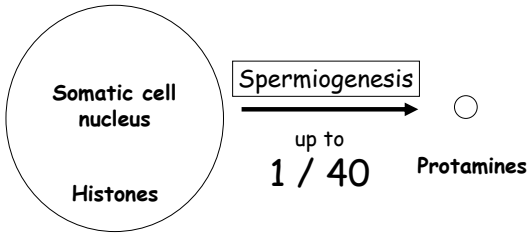


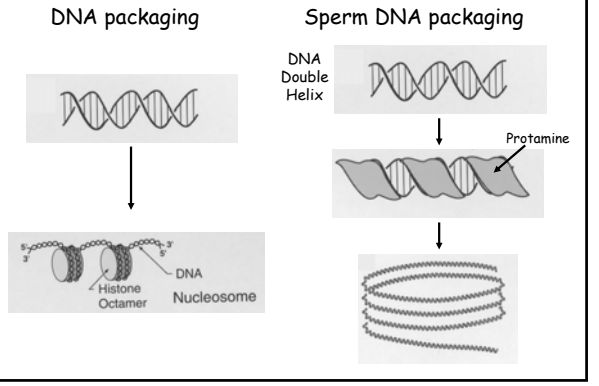
PARP1



APE

Mammalian Sperm nuclear compaction

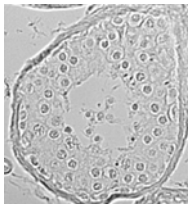


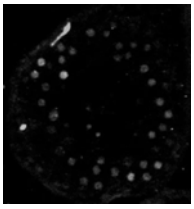


Topoisomerases

- Topoisomerases are nuclear enzymes that remove torsional stress in DNA.
- During meiosis and mitosis their function is important for:
  - replication
  - transcription
  - chromosome condensation
  - chromosome segregation

Topoisomerase IIα expression in human testes

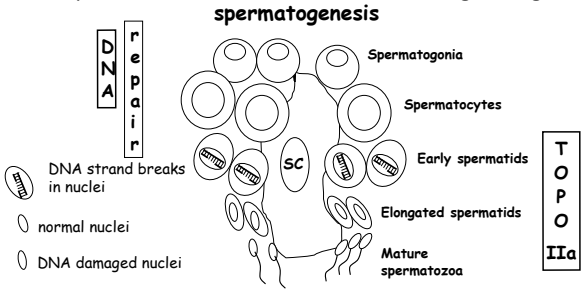




Topoisomerase

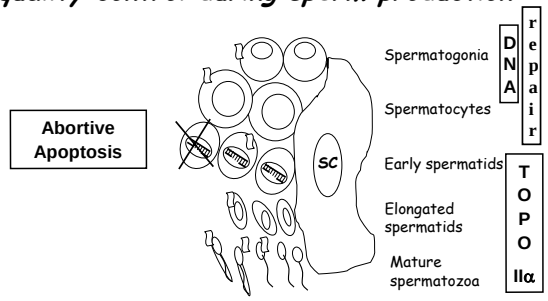
- Topoisomerase IIa may mediate the creation and ligation of DNA strand breaks during chromatin condensation in male germ cells.
- We have speculated that the increase in DNA strand breaks observed in ejaculated sperm of men with abnormal semen parameters may be caused by inadequate repair of DNA strand breaks introduced during chromatin condensation.

A putative model for the role of DNA repair and Topoisomerase IIa in nuclear remodelling during spermatogenesis



WHEN CHALLENGED EXTENSIVELY NATURE APPEARS UNABLE TO COPE WITH THE SELECTIVE DEPLETION OF ABNORMAL SPERM CELLS AND PRODUCES AN INCREASED PROPORTION OF ABNORMAL SPERMATOZOA

Failure of natures sperm selection quality control during sperm production



What do we see in some ejaculated spermatozoa?

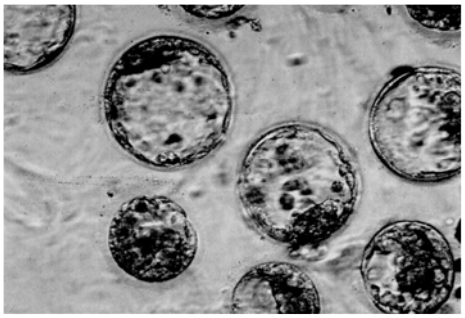
A. Chromatin packaging anomalies
B. DNA strand breaks
C. Presence of apoptotic marker proteins

How can we limit the effect of abnormal sperm on reproductive outcome?

Limiting harmful paternal influences

Using density gradient centrifugation techniques to routinely prepare sperm samples and limit the % of DNA damaged sperm

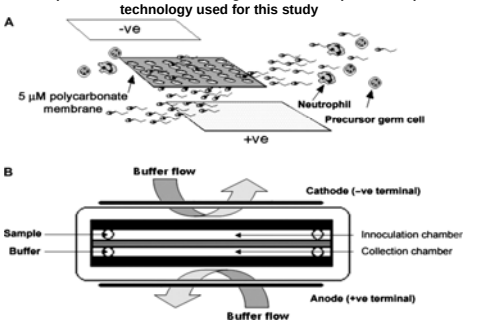
Culturing post-embryonic genome activation



Novel selection methods for enhancing sperm quality

- Aitken has reported a novel electrophoretic sperm isolation technique that produces suspensions containing motile, viable, morphologically normal spermatozoa which exhibit lower levels of DNA damage (Ainsworth et al., 2005).

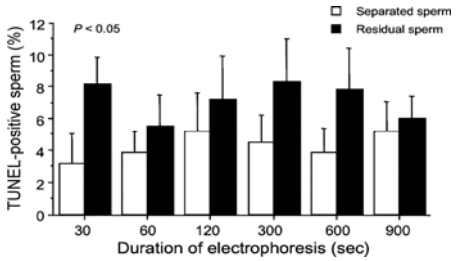
Schematic representations of the cartridge-based electrophoretic separation technology used for this study



Ainsworth, C. et al. Hum. Reprod. 2005 20:2261-2270; doi:10.1093/humrep/dei024

HUMAN REPRODUCTION

Impact of electrophoretic treatment on DNA integrity measured as percentage of TUNEL-positive cells (mean {+/-} SEM) for residual and separated sperm populations (n=6)



Ainsworth, C. et al. Hum. Reprod. 2005 20:2261-2270; doi:10.1093/humrep/dei024

HUMAN REPRODUCTION

Novel selection methods for enhancing sperm quality

- Selection of sperm under high magnification prior to ICSI (Bartoov et al., 2001).
- A technique using Magnetic cell sorting (MACS) using annexin V conjugated microbeads which eliminates apoptotic spermatozoa has also recently been reported (Said et al., 2005).

In vitro models showing the impact of magnetic cell separation on the sperm penetration assay (SPA) and sperm chromatin decondensation (SCD) following ICSI [Said et al. 2006]

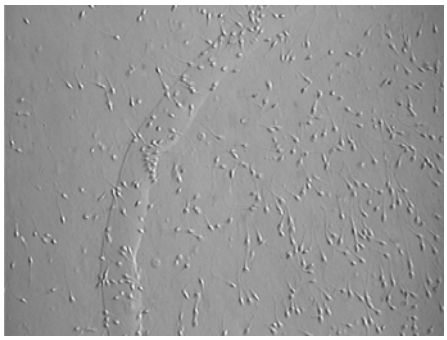
	SPA (n=16)		SCD (n=19)
	Oocytes penetrated (%)	Sperm Capacitation index	Decondensed sperm (%)
Annexin V-negative	44.5 ± 12.6*	1.8 ± 0.3*	34.2 ± 13.7*
Annexin V-positive	20.8 ± 5.3	1.3 ± 0.4	24.6 ± 13.0
Control	33.8 ± 6.9	1.5 ± 0.6	34.0 ± 13.0

Novel selection methods for enhancing sperm quality

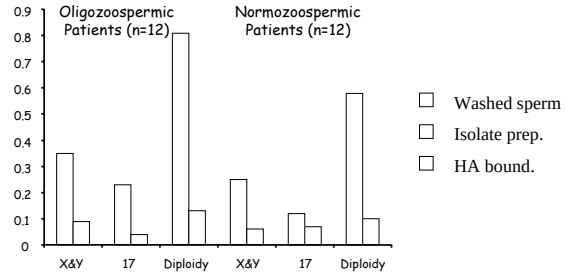
- Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status (Huszar et al, Fertil. Steril. 2003)

Selection of "normal" spermatozoa prior to ICSI using petri dishes coated with HA a polysaccharide whose receptor is present on the membrane of good sperm – Gabor Huszar Lab

(Jakab et al., 2005)

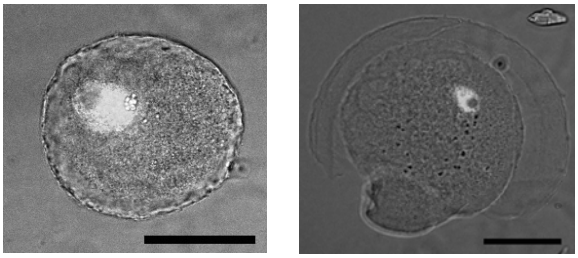


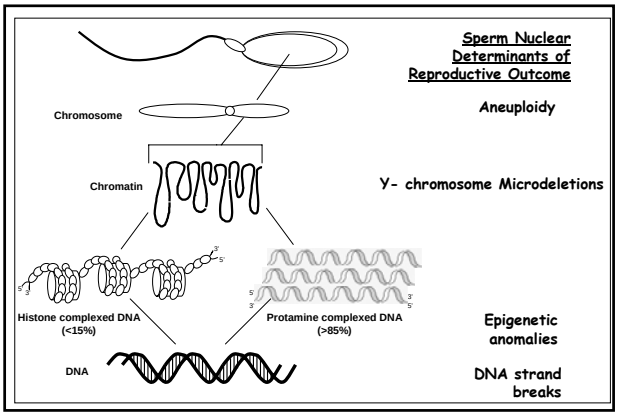
Selection of sperm with low aneuploidy frequencies (X, Y and 17 disomy) using HA – Gabor Huszar Lab

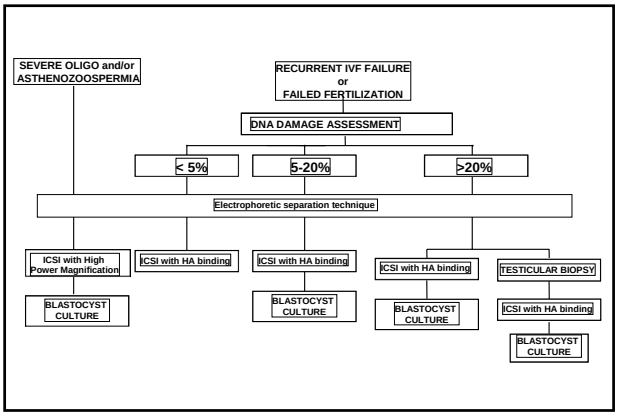


(Jakab et al, 2005)

The presence of the Base Excision Repair Protein [PARP1] in human oocytes. Can the egg take care of poor sperm DNA?







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Oocyte Selection

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Learning Objectves:

1. To obtain an overview of current methods and possible predictive markers to identify follicles with presumably good quality oocytes for fertilization or transfer after fertilization by IVF or ICSI.
2. To obtain an overview of markers used to select oocytes after analysis of morphology by conventional microscopic observation.
3. To be introduced into methodologies to non-invasively analyse spindle and zona pellucida by polarisation microscopy as markers of oocyte maturational state and developmental competence.

Summary:

Selection of oocytes for fertilization and transfer in IVF cycles mainly relies on morphological analysis of cumulus- oocyte complexes, microenvironment (e.g. components of follicular fluid), and markers of health and metabolism of granulosa cells to indirectly assess oocyte quality. In ICSI cycles it is mainly the morphology of the oocyte, its maturational status and chromosomal analysis of the first polar body (PB) that are currently used to assess oocyte quality and developmental potential. The non-invasive analysis by enhanced polarisation microscopy provides a novel tool to obtain information on presence or absence of a meiotic spindle, localisation of the spindle with respect to the first PB and high order and density of spindle fibres. These markers may relate to the oocyte's capacity to segregate chromosomes with high fidelity, to become fertilized and develop to a normal two-pronuclear zygote and contribute to a pregnancy. In addition, analysis of expression of a birefringent spindle may also contribute to optimise the time for ICSI, and in this way increase the number of high quality embryos. Furthermore, average birefringence of the zona pellucida was positively correlated to conception cycles, and may therefore be analysed for oocyte selection and improvement of treatment of patients attending the ART centres.

Introduction

Trisomy data and cytogenetic analysis of oocytes from younger and aged patients indicate that particularly those oocytes that have rested for extended times in the primordial follicles prior to resumption of maturation may have an increased risk for chromosomal errors, predominantly at first meiosis (reviewed by Pacchierotti et al., 2007). Furthermore, individual oocytes from cohorts obtained from the ovary in stimulated cycles may differ substantially in their developmental competence to become fertilized, support preimplantation development, zygotic gene activation and implantation of the embryo, and result a pregnancy and birth of a healthy child. Oocyte cryopreservation or oocyte maturation under sub-optimal conditions may increase risks for chromosome segregation errors or congenital abnormality. Selection of the best oocytes might reduce such risks. Currently, it is still unclear whether and to which

extend natural selection of a dominant follicle may contribute to ovulation of an oocyte with the best quality from the cohort of follicles initially induced to enter the growing stage *in vivo*. Hyperstimulation may bear risks for increasing the numbers of oocytes with chromosomal imbalance or low developmental competence (e.g. Baart et al., 2007). Also, it is still a matter of debate whether and in which way hormonal stimulation and different stimulation protocols may influence rate of maturation to meiosis II, and the chances to obtain one or many high quality oocytes with good developmental potential.

Ethical and legal constrains prohibit to select oocytes after fertilization for transfer in countries like Italy posing a considerable constrain to use time-requiring or postfertilization methods of analysis to identify high quality oocytes and embryos and assess chromosomal constitution. Therefore it is most important to identify parameters of follicle and oocyte morphology and physiology that can be assessed non-invasively and that are highly predictive of chromosomal constitution and developmental competence of the oocyte to improve pregnancy rate. Such scoring systems should not only increase success rate in the ART centre but may be also useful to reduce multiple embryo transfer and multiple pregnancy rate, and optimise treatment and maturation protocols to achieve pregnancy.

When considering crucial events in oocyte development that influence oocyte competence, it has to be kept in mind that oocytes acquire the capacity to resume and complete nuclear maturation initially during the long growth phase at folliculogenesis. During this time the oocyte and follicle diameter substantially increase. The oocyte chromatin is epigenetically modified to mediate mono-allelic gene expression in the embryo and offspring (for review see: Trasler, 2006). Finally, the oocyte also acquires developmental competence during this stage that is associated with the capacity to remodel the chromatin from the male gamete as well as mediate global alterations in chromatin configuration. Oocyte-derived factors are essential to initiate recruitment of stored mRNAs and complete full zygotic gene activation (e.g. Andreu-Vieyra and Matzuk, 2007). During oocyte growth there is a major increase in numbers of mitochondria and other cell organelles like sER. Many proteins, e.g. such of the zona pellucida, are actively synthesised to form an ordered three-dimensional meshwork of zona fibres as on outer extracellular shell around the oocyte, as well as gene products to mediate cytoskeletal reorganisation and control cell cycle progression. mRNAs are synthesised that may be either immediately translated or stored for timed polyadenylation and expression at maturation or during early embryogenesis (e.g. Su et al., 2007). From studies in experimental animals and transgenic models as well as analysis of gene expression at different stages of folliculogenesis it is well established that co-ordinated activity of the oocyte and the somatic compartment is required for normal oocyte and follicular development. This depends on direct intercellular communication via gap-junctions and auto- and paracrine signalling in which the oocyte appears to present the major regulator of its own metabolic activities and the differentiation and expression patterns in the somatic compartment (Eppig, 2005). Upon timely signalling through their cognate receptors gonadotrophs, prostaglandins (Ben-Ami et al., 2006), EGF-like molecules (Conti et al., 2006) as well as oocyte-derived growth and survival factors (e.g. Husein et al., 2006;) regulate oocyte growth and resumption of maturation, as well as remodelling of the extracellular matrix and ovulation in a complex temporally controlled fashion that is regulated by the oocyte-somatic cell-mediated dialog.

In assisted reproduction, oocyte selection usually has to take place right after retrieval when oocytes ideally have developed to the metaphase II stage. In order to identify high quality oocytes one would like to obtain as much information on the processes involved in the earlier

growth and maturation stages as possible to be able to select the best oocyte(s) for fertilization and to use single or multiple non-invasive markers in scoring to identify the presumably best embryo for transfer. In fact, the parameters that would be expected to have the highest predictive value would be those reflecting most accurately oocyte history including development prior to resumption of maturation. Rather than evaluating exclusively the correlations between oocyte morphological features (or the dysmorphic features) with respect to fertilization and embryo scores or to implantation and clinical pregnancy rate it appears therefore important to consider which parameters might be intimately related to earlier events in oocyte development and reflect normal or disturbed oocyte growth and maturation and chromosomal constitution. Ideally, markers should be easily identified and handling should be non-invasive and applicable to comply with routine schedules in the IVF laboratory.

Methods used for Oocyte and Follicle Selection

Traditional methods for the evaluation of oocyte quality are mainly based on morphological classification of the follicle, the cumulus-oocyte complex, and maturational state of the oocyte (germinal vesicle breakdown/meiosis II) and PB morphology. By now it also became possible to screen for presence and localisation of the meiotic spindle and assess the structural integrity of the zona pellucida non-invasively by qualitative and quantitative polarisation microscopy (Keefe et al.,2003; Eichenlaub-Ritter et al., 2004; Rienzi et al., 2005). Chromosomal analysis of the first PB offers a way to obtain information on the chromosomal constitution of the metaphase II oocyte although it is not possible to predict second meiotic errors. With the genesis of sensitive methods to perform molecular analysis of gene expression and of functional integrity of cumulus cells and components in follicular fluid (FF) it appears now also feasible to identify high quality oocytes indirectly and develop standard protocols for culminate scoring strategies with high predictive value.

Features associated with an optimal oocyte environment and good quality of individual follicles

Markers in follicular fluid

The health of the oocyte and follicle may be reflected by the accumulation of components in the follicular fluid (FF). A large number of studies focused on identifying markers for optimal microenvironment during/after oocyte maturation in the follicular fluid. For instance, concentration of anti-muellerian hormone (AMH) in serum have been used to assess follicular store as this might help to predict reproductive age, response to stimulation and chances to conceive. Recently it was shown that AMH in follicular fluid was positively correlated to embryo implantation while fertilization rate and embryo morphology was not affected by AMH concentration (Francin et al., 2007). Furthermore, colour, pulsed Doppler ultrasound analysis of individual preovulatory follicles was initially suggested to provide an indirect index of the oocyte's developmental competence within a highly vascularised follicle. However, the predictive power of this parameter has been challenged by several recent studies, and even those studies finding positive correlations caution against the rather limited clinical application of the method (Paffoni et al., 2006; Ragi et al., 2007). FF leptin levels appeared to present a good predictor of oocyte fertilization rate and was superior to measurements of follicular diameter while data on leptin and implantation and pregnancy rate are still missing (DePlacido et al., 2006). High IGF-II, IGFBP-3, IGFBP-4, and low PAPP-A levels in FF at the time of oocyte retrieval suggested better oocyte maturation and early embryo development (Wang et al., 2006), and thus may be used to select oocytes for in vitro fertilization. High bone morphogenetic protein-15 level in follicular fluid appeared also to be

associated with presence of a high quality oocyte and subsequent embryonic development (Wu et al., 2007), similar to high lactoferrin concentrations in FF that correlated with both a high fertilization rate and good embryo quality (Yanaihara et al., 2007). Unfortunately, many of the methods involved in analysis of FF rely on selective collection and quantification of components from FF in individual follicles. Only when the methodology can be standardized to be easy, fast and cheap they appear useful for routine oocyte selection. Moreover, much of the currently available information comes from retrospective studies, which frequently assessed fertilization, embryo morphology or development to the blastocyst while it is less clear whether there is a tight link to implantation and pregnancy rate.

Cumulus-related predictive parameters

Since it is the oocyte that is governing gene expression and differentiation of the granulosa cells within the follicle, several studies focused on identifying markers of good follicles, and hence, high quality oocytes by analysis of gene expression, and activity/apoptosis of cumulus cells (e.g. Corn et al., 2005). Expansion and mucification of the cumulus are hallmarks of a fully mature follicle Mature, grade 3 cumulus-oocyte complexes (COCs) appear associated with higher fertilization rate as well as development to blastocyst of good quality in IVF cycles (Corn et al., 2005). Apart from scoring morphology of the cumulus-oocyte complex (COC), expression of components of extracellular matrix might be expected to have predictive value. In fact, the expression of PTGS2 and HAS2 was 6-fold higher, and that of GREM1 was 15-fold higher in human cumulus yielding higher grade embryos versus lower grade embryos (McKenzie et al., 2004). Soluble pentraxin-3, another structural constituent of the cumulus oophorus that stabilises the extracellular matrix was elevated in follicular fluid after stimulation (Salustri et al., 2005) but concentrations of PTX3 in FF did not apear to be related to oocyte quality (Paffoni et al., 2006).

In conclusion, there are retrospective studies showing correlations between expression of genes in cumulus, apoptosis of cumulus and gene products in follicular fluid and fertilization rate, embryo quality and/or implantation and pregnancy in assisted reproduction cycles. The future has to show the predictive value in prospective randomised studies and has to prove that the assays can be performed easily, cost effectively and in a brief period of time to be useful in oocyte selection.

Oocyte predictive markers

Oocyte nuclear maturation status

Oocyte maturational status can be assessed by presence of a PB - so far it has been assumed that oocytes that underwent cytokinesis all have developed to metaphase II. This notion has been challenged by spindle analysis by polarisation microscopy. The latter revealed that some oocytes with PB are still in telophase I or prometaphase II of nuclear maturation (Eichenlaub-Ritter et al., 2002; Montag et al., 2006). Culture for some hours appears to provide time for progression to metaphase II suggesting that it is possible to increase the number of healthy, fertilization competent oocytes for selection by performing ICSI on these at a later time after retrieval (Montag et al., 2006). We observed that only a low percentage of germinal vesicle (GV)-stage immature oocytes retrieved in ICSI cycles possessed a birefringent spindle after *in vitro* maturation to meiosis II and emission of a first PB. In support of a limited potential of these oocytes to form a functional spindle and segregate chromosomes with high fidelity at meiosis I, recent analysis of PB chromosomal constitution by fluoresecent *in situ*

hybridisation (FISH) with chromosome specific probes highlight the high rate of aneuploidy in such *in vitro* matured oocytes compared to the mature controls (Magli et al., 2006).

Oocyte chromosomal status

While some patterns of pronuclear morphology are associated with a higher proportion of euploidy and implantation reaffirming the relevance of this scoring system for the prediction of zygote viability (Gianaroli et al., 2007), there is up to now no conclusive evidence that any individual oocyte dysmorphic feature is a good predictive indicator of chromosomal constitution (Chamayu et al., 2006). However, over half of the embryos derived from oocytes with centrally granulated cytoplasm were aneuploid (Kahramann et al., 2000). Aneuploidy rate was also increased in oocytes with cytoplasmic abnormalties compared to those with normal morphology or extracytoplasmic aberrations (reviewed by Balaban and Urman 2006). Analysis of the first PB and also second PB e.g. by FISH with chromosome-specific probes can be used to identify aneuploid oocytes with normal or aberrant morphology and select those with presumably euploid constitution for fertilization or transfer, respectively. Unfortunately, time required for the analysis necessitates that oocytes are frozen before fertilization in countries like Italy, posing risks for damage of the spindle and possibly increased susceptibility to second meiotic errors after the freezing/thawing procedure and fertilization. It is also still a matter of debate whether oocyte selection after PB analysis will result in increased implantation and birth rates (for discussion see: Shahine and Ceddars, 2006). Keeping in mind that there may be the risk of false-negative results that increases with the numbers of chromosome-specific probes, it appears important to consider the age of the patient and the number of oocytes in each individual cycle to evaluate risks and benefits of the method for oocyte selection.

Dysmorphic oocyte features

The list of dysmorhic markers suggested to be associated with reduced quality and developmental potential of the oocyte and embryo is long.

Giant oocytes are characterized by possessing twice the number of chromosomes, possibly as a result of polyploid oogonia or fusion of two oocytes. We observed two spindles in such oocytes. Independent of whether these oocytes emit one or two PBs after fertilization, the embryo will be polyploid (for references see: Ebner et al., 2006) and cannot develop normally. Giant oocytes should therefore not be considered for selection in assisted reproduction.

Minor dysmorphisms such as refractile bodies in ooplasm appear to have no pronounced effect on fertilization and embryo quality in ICSI patients (for review see: Ebner, 2006), while the presence of vacuoles in ooplasm was found to reduce fertilization rate in one ICSI study. Whether or not disturbances occur may mainly depend on the size of the vacuole.

Presence of sER clusters may be indicative of a disturbance affecting embryo development since clinical pregnancy rate was much lower in oocytes without compared to those with sER clusters. In addition, a baby born from a cluster-positive oocyte that was fertilized had Beckwith.Wiedeman syndrome, a disease associated with disturbed expression from an imprinted gene (for reference see: Ebner et al., 2006; Balaban and Urman, 2006).

There is a long debate on the predictive value of the size and morphology of the first PB. In a study by Ebner et al. oocytes with a normal sized, intact first PB yielded embryos with higher rates of conception cycles. Those oocytes with fragmented PB had significantly decreased

blastocyst formation rate (for references see: Ebner et al., 2006; Balaban and Urman, 2006). Since fragmentation rate is likely to increase over time post-retrieval (Eichenlaub-Ritter et al., 1995), this may explain the negative results obtained by two other studies in which the timing of ICSI and analysis of PB morphology differed (for references see: Ebner et al., 2006; Balaban and Urman, 2006). There was no correlation between PB shape and chromosomal constitution of PBs in oocytes of aged patients (Verlinsky et al., 2003). Mouse models suggest that very large PBs are a hallmark of loss of cell cycle control and disturbed spindle function (Verlhac et al., 2000). Future studies have to reveal if this is also true in the human. The comparatively rapid degeneration of the first PB and possible differences in maturation kinetics associated with maternal age and stimulation protocols make it difficult to unambiguously assess the power of PB scoring for oocyte selection.

Clear, colorless cytoplasm is considered as marker of good quality in oocytes while extensive cytoplasmic granularity, particularly central granulation, was negatively correlated to a conception cycle and was associated with increased aneuploidy in embryos (Kahraman et al., 2000). The presence of a dark cytoplasm decreased by 83% the likelihood of obtaining a good quality embryo and thus may be considered negatively correlated with oocyte quality (Ten et al., 2007). Increased cytoplasmic viscosity is also considered as marker of low oocyte quality but is difficult to quantify objectively (for reference see: Ebner et al., 2006). Funnel persistence as a result of high viscosity can only be analysed in ICSI cycles after microinjection of sperm and can thus not be used for oocyte selection before fertilization.
Oocytes with a larger than normal perivitelline space were associated with 1.8 times higher chance of having good quality embryos compared to those with perivitelline dysmorphisms (Ten et al., 2007). Perivitelline granularity was not predictive of fertilization and cleavage rate but may rather be a physiological maturation-dependent phenomenon related to stimulation protocol (for references see: Balaban and Urman, 2006).

Presence, integrity and localization of the meiotic spindle
Analysis of spindle morphology in fixed human oocytes suggests that oocyte ageing is frequently associated with presence of a distorted, aberrant or small spindle with unordered chromosomes. Postovulatory ageing also results in gradual degeneration of the spindle- both indicating that there is a high risk of errors in chromosome segregation (Eichenlaub-Ritter et al, 2004). Spindles can by now be qualitatively and quantitatively analysed in a non-invasive fashion in living human oocytes using enhanced polarisation microscopy employing circularly polarised light and electronically controlled liquid crystal compensator optics. This method has been used to analyse presence and localisation of the second meiotic spindle in human oocytes, and quantify light retardance as this is directly dependent on microtubule number and high order algnment of spindle fibres (for references see: Shen et al., 2006). Consistently, oocytes that did not contain a birefringent spindle had lower fertilization rates (for references see: Shen et al., 2006). The analysis also showed that some of the oocytes that possessed a PB were still in telophase I, and some presumably in prometaphase II (Eichenlaub-Ritter et al.,2002; Montag et al., 2006). Culture for one to two hours increased the number of spindle-positive oocytes. The accurate assessment of maturational stage can improve optimal timing of ICSI and oocyte selection to improve pregnany rate. The usefulness of the mere analysis of presence or absence of a spindle may still be limited since more than 80% of all oocytes possessed spindles in most recently published studies. Of those without spindle the majority was fertilized and progressing to the two-pronuclear stage in our hands. Although one study including a large cohort of oocytes and cycles failed to find a significant correlation between presence of a birefringent spindle and pregnancy and implantation rate (Chmayou et al., 2006), less than 50% of all oocytes retrieved and used for transfer after ICSI possessed a

birefringent spindle. The technicians might have missed to detect the spindle in many of the large cohort due to lack of experience or time, or sub-optimal handling or low oocyte qualities might be the reason. The low percentage of spindle-positive oocytes casts therefore some doubts on the reliability of the data.

Since only about 10-20% of all meiosis II oocytes might miss to express a birefringent spindle under optimal conditions, there is still a dilemma to chose the best oocyte from the spindle-positives. Most oocytes have the spindle located close to the first PB (e.g. Shen et al., 2006). There are several studies that suggest that severe spindle displacement might be an indicator of reduced oocyte quality. Rienzi et al. (2003) found that the meiotic spindle of *in-vitro* matured oocytes was nearly always aligned with the first PB, suggesting that the misalignment observed in the oocytes matured *in vivo* results from a displacement during the manipulations for the cumulus and corona removal. It is still unclear whether scoring right after retrieval, after cumulus removal, or at ICSI may also influence the overall frequency of spindle displacement. Generally, one may avoid selecting oocytes with severe spindle displacement when there are sufficient additional oocytes in the treatment cycle. Experimental models provided evidence that adverse exposures affecting microtubule polymerisation or spindle shape could be visualised by polarisation microscopy (Eichenlaub-Ritter et al., 2007; Shen et al., 2005), such that recurrent occurrence of dysmorphic spindles in oocytes of individual patients might reflect disturbances by acute or chronic exposures, unsuitable treatment protocol, or problems related to presence of polymorphisms and mutations. Polarization microscopy showed that current techniques of oocyte cryopreservation may cause meiotic spindle destruction.in human oocytes (Rienzi et al., 2005). It appears therefore sensible to select those cryopreserved oocytes with spindle for ICSI although prospective studies on the predictive value of the scoring are still missing.

Since the majority of oocytes in most ICSI cycles involving patients of fairly young reproductive age do possess a spindle, the predictive value of quantitative spindle analysis may be limited. By contrast, the robustness of a spindle appears to be a much more useful predictive marker of oocyte quality. The retardance of light by Polscope microscopy is linearly related to numbers of microtubules (for reference see: Shen et al., 2006). In fact, the mean retardance of light by the birefringent spindle of oocytes forming a pre-embryo with good PN score after ICSI was significantly higher compared with spindles of oocytes developing into a lower PN score pre-embryo with limited developmental potential ($P < 0.001$) (Shen et al., 2006). Since 2-PN embryos with high scores appear to be less frequently aneuploid compared to those with mediocre or low scores (Gianaroli et al., 2007), the selecion of oocytes with spindles of high retardance may also help to improve pregnancy rate and reduce rates of spontaneous abortions as a consequence of chromosomal aberration. In the retrospective study we showed that transfers involving oocytes with high retardance and at least one good PN score embryo resulted more frequently in a conception cycle than transfers from oocytes with spindles of lower mean retardance and lower PN score embryos (Shen et al., 2006). From the data it appears that quantitative analysis of the spindle by Polscope largely reflected the PN-scoring and may therefore be substitute for PN-scoring in countries like Italy.

Quantitative analysis of the zona pellucida

The zona pellucida is one of the cellular constituents of the oocyte that is synthesised during oocyte growth and maturation. The relative order of fibres might reflect a continuously favourable environment during a period of oocyte development when it acquires nuclear and cytoplasmic maturational competence. Some studies have focussed on measuring zona

thickness but differences between groups were always fairly low and therefore not very useful to select oocytes. We analysed retrospectively the mean retardance of light by the three layers of the zona pellucuda in line scans across the zona from oocytes transferred after ICSI. The study showed that the mean magnitude of light retardance was nearly 30% higher ($P < 0.001$) in the inner layer of the zona pellucida of oocytes contributing to a conception cycle compared to a non-conception cycle (Shen et al., 2005b). The magnitude of light retardance by the zona pellucida inner layer appears therefore to present a unique non-invasive marker for oocyte developmental potential and/or cycles with high probability of success. A recent study employing the same techniques fully confirmed the findings by showing a correlation between mean retardance of the human oocyte spindle and zona and developmental potential of the oocytes (Rama et al., 2006). It appears now important to develop imaging systems that facilitate quantitative evaluation and thus can be used in routine clinical application.

Conclusions:

The evaluation of the predictive power of analysis of components in FF and in cumulus survival and expression profiles still awaits further confirmation in prospective, randomised studies. Considering the rapid improvements of methodologies for expression studies, the assessment of health and quality of cumulus and the somatic compartment of the follicle bear promises to improve oocyte selection. While the predictive value of scoring of individual oocytes according to morphological features for oocyte selection is still debated, culminate scoring criteria may prove to be valuable. Taking note of the recurrent occurrence of dysmorphisms in oocytes can be helpful to adjust hormonal stimulation protocols, and counsel patients. PB analysis for chromosomal aberrations appears particularly useful when it is possible to select at the pronuclear stage, provided sufficient numbers of oocytes are available. Quantitative polarisation microscopy presents a novel tool to assess not only presence and localization but also high order structure of cellular constituents like the spindle and the zona pellucida of the oocyte. Due to its non-invasive nature this technique appears to offer a unique opportunity to greatly improve oocyte selection prior to fertilization, and when combined with appropriate software for rapid and reliable analysis could become the choice method of oocyte selection in ICSI cycles. Furthermore such methods should be invaluable to improve oocyte handling and maturation in assisted reproduction, and possibly identify the aetiological factors in reduced fertility.

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Selecting embryos

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Selecting embryos with high implantation potential is one of the most important challenges in the field of assisted reproduction. Moreover it is important to identify viable and non-viable embryos in order to cryopreserve only the former and to discard the latter. Such identification needs to be accurate in order to avoid massive and useless cryopreservation and to improve the chances of achieving a pregnancy for IVF couples. Various methods have been proposed to evaluate embryo viability in IVF programmes. A limiting factor in such evaluations is that they should be neither invasive nor time-consuming.

Which usual day 1/2 parameters to select embryo?

Zygote scoring system on day 1

Studies on pronuclear morphology on day 1 have reported a wide range of observation times mostly between 16 and 18 hours post insemination/ICSI. Many morphological transformations can easily be assessed during the development of human pronuclear zygotes by simple non-invasive microscopic observation. Zygote scoring systems on day 1 have been reported to be reliable in terms of viability and implantation. Based on empirical observations correlated with pregnancy and previously published observations of zygote morphology, a zygote scoring system was first proposed by Scott and Smith in 1998. A strong correlation between this score and the implantation rate was reported. This initial zygote scoring system was later revised (Z1 to Z4 score) to allow faster scoring and to make it easy to use by a large number of technicians (Scott *et al.,* 2000). At the same time, a further group proposed another classification based on static observation of human zygotes (Tesarik and Greco, 1999). Six patterns of pronuclear morphology (pattern 0 to 5) related to zygotes that had equal-sized pronuclei in apposition were described. The normal range of pronuclear variability was defined by analysis of zygotes giving rise to embryos transferred in 100% implantation cycles (pattern 0). Since this date, many different pronuclear scoring systems derived from these classifications have been proposed to select high-quality embryos.

Early cleavage on day2

This evaluation is performed in most IVF centres 25-27 hours post insemination/ICSI. Early-cleavage embryos on day 2 were reported to achieve higher implantation rates compared to non-early cleavage embryos. The reasons for better viability of early cleavage embryos remain largely unexplained, but it may be speculated that such embryos derive from oocytes with adequately synchronized cytoplasmic and nuclear maturation. Early cleavage embryos should be less likely to be exposed to a critical minimal level of maternal mRNA prior to activation of the embryonic genome.

Embryo morphology on day 2/3

Selection of the most viable cleavage stage embryo is usually based on embryo morphology on day 2/3, with the rate of development in culture as a guide (for a review: see Ebner *et al.,* 2003). Embryo morphology is determined by the number, size and shape of blastomeres, the

proportion of fragments and the presence of multinucleated blastomeres. Blastomere size is a relevant characteristic that is generally underestimated. When comparing day 2 transfers between 4 cell embryos, it was found that unevenly cleaved embryos had significantly lower implantation rate compared with evenly cleaved embryos. Integration of this criteria remains complex as it is related to the number of cells. It has been demonstrated that after 2 days of culture, the 4-cell stage is the optimal cleavage stage. Embryos at this cleavage stage with little or no fragmentation and without multinucleated blastomeres are associated with a higher implantation rate compared to embryos at other cleavage stages with fragmentation or multinucleated blastomeres.

Day 1/2 parameters and blastocyst development

Although the best way to evaluate embryo viability is its ability to implant, assessment of embryo development in vitro might make it possible to remain independent of the uterine receptivity that clearly influences implantation success. Extended embryo culture until the blastocyst stage (day 5/6) was successfully proposed as a means to select the most developmentally competent embryos. Delaying transfer until the blastocyst stage should increase the potential for self-selection of viable embryos since only a proportion of embryos reach this stage; the embryonic genome being activated between the four-and eight-cell stage. When embryos are cultured in vitro, about 50% will cease development during the first week. The reasons for this high rate of embryo loss during early development are not fully understood. They might include chromosomal abnormalities, suboptimal culture conditions or inadequate oocyte maturation.

Zygote scoring system and blastocyst development

Arrest of development beyond day 2 was most frequently associated with non-pattern 0 zygotes (Tesarik and Greco, 1999). Moreover, embryos derived from the Z-1 score, which is included in pattern 0 in Tesarik's classification, resulted in a higher percentage of day 5/6 blastocysts: 76% on day 6 (Scott *et al.,* 2000). Such results were confirmed using other detailed descriptions of zygote morphology and scoring systems (Neuber *et al.,* 2003).

Early cleavage and blastocyst development

It was reported a higher blastocyst rate with early cleavage embryos than with uncleaved embryos (ie: Day 5: 39% vs 21%, respectively) (Neuber *et al.,* 2003). A positive correlation between early onset of cleavage and blastocyst formation had already been observed in mouse and bovine embryos. In these studies the blastocysts with early cleavage were found to have more cells than their later cleaving counterparts. Such results are better explained by the differences in timing of the first cleavage rather than differences in the rates of progression of subsequent cell cycles.

Embryo morphology on day 2/3

There is evidence that embryo selection on day 2/3 based on morphological criteria may be unreliable, resulting in the transfer of embryos that are abnormal or arrested at later developmental stages. With regard to the embryo morphology on day 2, the blastocyst rate is higher for 4-cell embryos compared to faster (5-8 cell) or slower (2-3 cell) embryos. Indeed, the presence of more than four cells at day 2 is not predictive of a greater likelihood of blastocyst formation. Two hypotheses have been suggested to explain this: (i) some blastomeres in these embryos might be rather large anucleate fragments, thus explaining the overestimation of their numbers and (ii) accelerated division might be an indicator of developmental instabilities that would affect the embryo's ability to develop to a blastocyst.

Fast-developing embryos have been reported to exhibit higher levels of aneuploidy compared to synchronous embryos.

The rate of blastocyst formation decreases significantly with fragmentation on day 2. Several hypotheses have been suggested to explain the detrimental effects of fragments on embryo development. First, fragments might physically impede cell-cell interactions, interfering with compaction, cavitation and blastocyst formation. In addition, ultrastructural observations of degeneration in blastomeres adjacent to fragments suggest that fragments might release toxic substances and therefore damage nearby cells. Alternatively, fragments might also reduce the volume of cytoplasm and deplete the embryos of essential organelles or polarized domains. Higher aneuploidy rates were reported for >35% fragmented embryos whereas it was also speculated that in the case of moderate fragmentation, various temporal or spatial patterns of fragmentation have quite different effects on embryo development than the occurence of fragments *per se*.

Combination of day1 to day 3 parameters and blastocyst development

The various parameters suggested to date to evaluate embryo viability have mostly been investigated as a group together rather than as individually cultured embryos. The possibility of combining or integrating findings from observations at different periods of time to improve the assessment of embryo viability has hardly been studied (Lan *et al.,* 2003; Neuber *et al.*, 2003; Rienzi, 2005). Using pronuclear morphology combined with day 3 morphology, Lan *et al.* (2003) observed 92% survival at day 5 in 499 top-quality embryos (Z-1 zygotes and grade I embryos). Another study, based on 1550 individually cultured embryos, explored various scenarios to predict blastocyst development from sequential embryo assessment (Neuber *et al.,* 2003). With pronuclear symmetry, early cleavage and subsequent good quality $\geq$4 and >7-cell embryos on days 2 and 3, respectively, they could predict 54% probability for an embryo to reach the blastocyst stage by day 5. Using a multiple-step scoring system, Rienzi *et al.,* (2005) reported 77% blastocyst formation on day 5 for embryos with normal pronuclear stage and early cleavage on day 1, 4-to 5-cells with equal blastomere size, <10% fragmentation and no multinucleation on day 2, and > 6 cells with equal blastomere size, <10% fragmentation and no multinucleation on day 3.

However the degree of predictability of parameters for non-top-quality embryos remains a major problem, rather than the outcome of the best embryos that combine all the best suited parameters, since unsuitable embryos according to day 1 and day 2 criteria might develop to the blastocyst stage. In our center, an extended individual culture of more than 4000 embryos was performed therefore first to assess the impact of pronuclear morphology, early cleavage, and embryo morphology on ability to develop to the blastocyst stage at day 5/6, then to evaluate their impact on blastocyst morphology and their implantation ability whenever accessible. Stepwise logistic regression analysis gave us the opportunity to determine the respective weighting of each parameter. This methodology indicated (i) that each of the four parameters studied influenced the blastocyst rate at day 5/6, with a greater influence of early cleavage and embryo morphology at day 2 on this outcome, and (ii) that only early cleavage and embryo morphology at day 2 had a significant relationship with regard to blastocyst quality. Altogether, these findings suggest a limited contribution of pronuclear morphology and fragmentation rate compared to early cleavage and embryo cell number. Moreover, even the combination of all four parameters led to an area under the receiver operating characteristics curve (AUC_{ROC}) of 0.688. Such a weak AUC_{ROC} highlights the limits of the early morphological parameters available to predict embryo viability. Similarly, when we look at the extended cultures that were achieved during the same study period for the purpose

of single blastocyst transfer, it is important to note that selection of the "right" embryo at day 2 would have been correct in 17%, possible (among various embryos of similar characteristics) in 26%, but incorrect in 52% of cases or impossible (no blastocyst development) in 5% of cases, in agreement with previous reports which observed that day 3 morphology was a poor predictor of blastocyst development in extended culture. Bearing in mind that embryo viability is best defined as the ability of the embryo to implant successfully and result in a normal healthy baby, we checked the issues of blastocyst transfers following extended culture of whole cohorts. As expected, transfers of good morphology blastocyst(s) were associated with higher live birth and implantation rates compared to all other blastocyst transfers. Interestingly, once looking at implantation ability of at least one good morphology blastocyst, the studied parameters of early embryo development revealed to be unhelpful to predict implantation.

Conclusion

Sequential morphological assessment of pronuclear morphology, early cleavage and embryo morphology on day 2 (number of cells and fragmentation rate) may all be considered as parameters related to development potential. However early cleavage and cell number contribute most to this evaluation. Moreover, the predictive power of pronuclear morphology, early cleavage, number of cells and fragmentation rate remains quite low, thus arguing for either additional evaluation of already assessed criteria (first polar body morphology, cytoplasmic halo, blastomere size, position of fragments) or definition of new criteria.

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