Workshop on QMS in FISH PGD

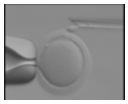
Culture, biopsy and spreading – - key points of the embryological procedure in PGD cycle

Gabriela Tauwinklová et al.

Types of cell to biopsy

- **First and second polar body**

(Verlinsky et al., 1996)
- Only permits diagnosis of female-related defects

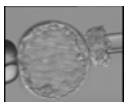


- **Blastomere biopsy from day-3 embryos**

(Tarin and Handyside, 1993)
- Used for any type of PGD indication

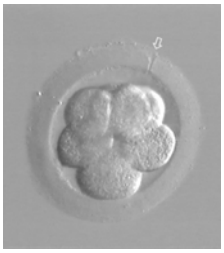
- **Blastocyst biopsy of trofectoderm cells**

(De Boer, 2004)
- The clinical application is very recent, limited data have been reported



Zona opening

- **Methods**
  - Chemical (not recommended for PB biopsy)
  - Laser
  - Mechanical
- **Only one breach in the zona**
- **Zona opening should not be**
  - too small (embryo squeezing during biopsy)
  - large (risk of loosing cell during manipulation)



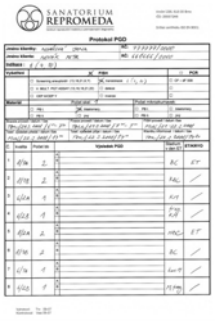
Cell removal

- **PB**
  - Aspiration
- **Cleavage stage biopsy**
  - Aspiration
  - Expulsion (by exerting pressure against the zona)
  - Displacing (through the zona opening with the flow of medium)
- **Blastocyst**
  - Herniation followed by laser
  - Mechanical excision
  - Mechanical stitch and pull method

Identification system in PGD cycle

- Until the time of biopsy, routine IVF culture conditions are applied
- Following biopsy single embryo culture is compulsory to ensure easy identification of embryos post-diagnosis
- In one moment gametes and embryos from only one couple are processed
- In one moment only one embryo is biopsied
- In one moment cells from only one embryo are fixed

Identification system in PGD cycle



Identification system in PGD cycle

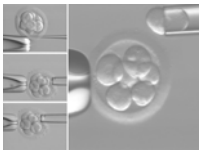
Special forms from IVF lab should accompany the samples, if the genetic analysis is not to be performed in the same lab which performs biopsy.

Following should be communicated to PGD lab:
- **Patients identification**
- **Type of genetic analysis which has to be done**
- **Correspondence between cells and embryo identification**

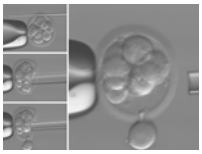
Embryo biopsy procedure

- **Embryo biopsy dishes are prepared in advance of the procedure**
- **Two (or more) incubators to minimize opening and closing the incubator, and hence producing temperature fluctuations are used**
- **Biopsy should be performed as quickly as possible**
- **Special care should be taken to avoid damage to the embryo during procedure!**

Mechanical embryo biopsy methods



- **Aspiration method**
  T-shape opening in the zona is followed by aspiration of the cell into a biopsy pipette



- **Expulsion method**
  includes cut in the zona and cells expulsion through zona opening by external push on the zona pellucida with the micropipette

Aspiration versus expulsion method

Lysis rates during biopsy

	Aspiration	Expulsion	
No of biopsied embryos	407	450	
No of removed blastomeres	631	784	
No of intact blastomeres	592	762	
No of cells lysed during embryo biopsy	39 (6.2%)	22 (2.8%)	P = 0.05

Outcome of pregnancies

	Aspiration	Expulsion	Aspiration + expulsion	
No of embryo transfers	19	30	25	
Clinical pregnancies	7	12	12	
Pregnancy rates (%)	36.8	40.0	48.0	NS
No of embryos replaced	33	55	50	
No of embryos per embryo replacement	1.7	1.8	2.0	
Ongoing implantations	9	16	15	
Implantation rates (%)	27.3	29.0	30.0	NS

- Aim: evaluate the influence of the use of aspiration biopsy method on the results of the biopsy procedure, further embryo development in vitro or the embryo replacement outcome, compared with expulsion biopsy method.
- Expulsion biopsy method is less time consuming (95.4s versus 48.2s, P<0,001) although the time difference has no impact on results
- Higher survival rate for the biopsied cells was found after expulsion method
- The percentage of embryos developed into the blastocyst stage as well as outcome of PGD cycles was very similar for both methods

(Data from our centre presented at ESHRE 2008)

Embryo selection - excluding criteria

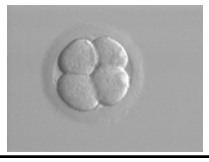
- Embryos that have not entered the third cleavage division (4 cells and less)

Day 2	Day 3	FISH results
2 cells	2 cells	98% abnormal
2 cells	3 cells	92% abnormal
3 cells	4 cells	87% abnormal
4 cells	4 cells	92% abnormal

FISH results on day 3 embryos which were arrested in development in at least one blastomere, in relation to the stage on day 2 (Magli et al., 2007)

Day 3	FISH results
4 cells	74% abnormal
5-6 cells	76% abnormal
7-8 cells	50% abnormal
9 and more cells	78% abnormal

Chromosomal abnormalities and cellular stage 62 hours after insemination (Magli et al., 2007)



Embryo selection - excluding criteria

- Poor embryo quality: › 50% fragmentation

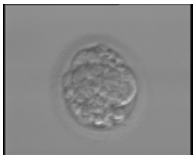
Fragmentation percentage has been associated with chromosome abnormalities (Plachot et al., 1987; Pellestor and Sele, 1988)

The percentage of fragmentation is correlated with mosaicism (Munné and Cohen, 1998)

Chromosomal abnormalities detected by FISH and fragmentation rate

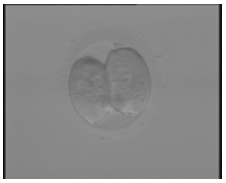
Fragments (%)	Aneuploid (%)	Mosaic and other (%)
0 - 15	11	29
20 - 40	8	47
45 - 100	11	89
	Not significant	P ‹ 0.001

Munné and Cohen, 1998



Embryo selection - excluding criteria

- **Poor embryo quality: multinucleated blastomeres (MNB)**



MNB usually are arrested cells, therefore embryos with one or more MNB are expected to be developmentally incompetent (Hardy et al., 1993)

The presence of MNB in non arrested day 2 or day 3 embryos is indicative in 74% of the cases of extensive mosaicism and/or polyploidy (Klingman et al., 1996)

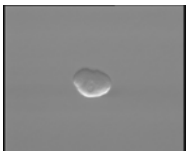
The correction of multinucleation after the second cleavage does not repair aneuploidy state of 4-cell human embryo (Hlinka et al., 2008)

Biopsy medium

PROS	CONS
BM facilitates the process of blastomere biopsy (Dumoulin et al., 1998)	Additional stressing factor for embryo
Lower risk of blastomere damaging during biopsy (Dumoulin et al., 1998)	Time limitation
Shorter time to perform biopsy is needed (Dumoulin et al., 1998)	Embryo has to be rinsed properly to remove traces of BM post biopsy

Cell selection

- **Identifiable cell**
  - **Distinct nucleus**
  - **Mononucleate cell**



- **Avoid**
  - **Cell, where nucleus is not visible (metaphase? anucleate cell?)**
  - **Multinucleate cell**

1 or 2-cell biopsy?

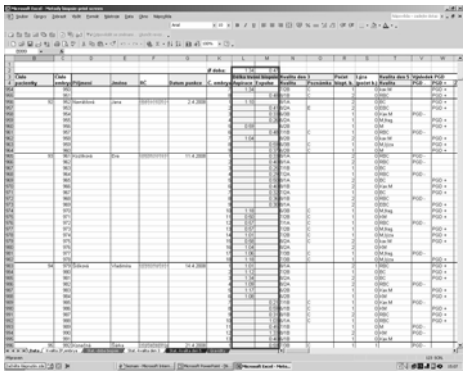
	1-cell removal	2-cell removal
8-cell	 0.125	 0.25
7-cell	 0.14	 0.29  0.25
6-cell	 0.16	 0.33  0.25
5-cell	 0.20  0.125	
4-cell	 0.25	

1 or 2-cell biopsy?

2-cell biopsy: PROS	2-cell biopsy: CONS
Day 3 developmental stage represents a stronger predictor for further development than the removal of 1 or 2 cells (Goossens et al., 2008)	Removing 1-c is less invasive than 2-c removal, thus resulting in more blastocysts on day 5 (Goossens et al., 2008)
In vitro development of good quality or more rapidly developing embryos is not impaired when 1 or 2 cells are removed (Baart et al.,2004; Van de Velde et al., 2000)	For FISH PGD cycles, 2-c biopsy does not increase the chance to obtain diagnosis (Goossens et al., 2008)
Biopsy of 1-c significantly lowers the efficiency of a PCR-based dg (Goossens et al., 2008)	2-c biopsy significantly impedes embryo development and is not advisable in cases in which PGD is being used for the purpose of increasing IVF success rates (Cohen and Wells, 2007)
Implantation rate and live birth rate is not significantly different after 2-c biopsy than after 1-c biopsy (Goossens et al., 2008)	

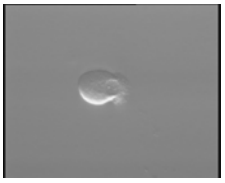
Speed of the biopsy

- Speed of the biopsy procedure is critical. It is recommended that one person does the embryo biopsy and the second one performs dish change-over of embryos (PGDIS guidelines)
- No specific recommendations for maximum time out of the incubator can be given.
- A documented record for biopsy timings is recommended (ESHRE PGD Consortium guidelines)



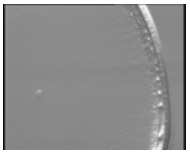
Cell lysis

- Integrity of the removed cell is extremely important for the correctness of the genetic analysis
- Changing pipette in case of lysis
- No lysed cell for PCR cycles



Spreading and fixation methods

- Methanol / acetic acid
(Tarkowski,1966; Munné at al., 1993)
- Tween / HCl
(Coonen et al., 1994; Harper et al., 1994)
- Combined Tween / HCl - methanol/acetic acid
(Dozortsev and McGinnis, 2001; Baart et al., 2004)



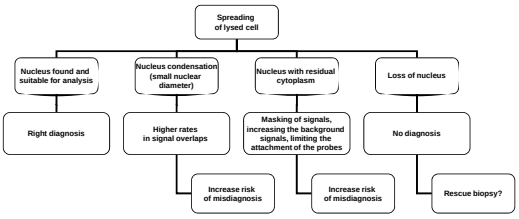
	No of embryos analyzed	No of embryos successfully diagnosed	Diagnostic efficiency (%)
2004	971	932	96.0
2005	1040	1022	98.3
2006	1113	1080	97.0

Spreading: What is important?

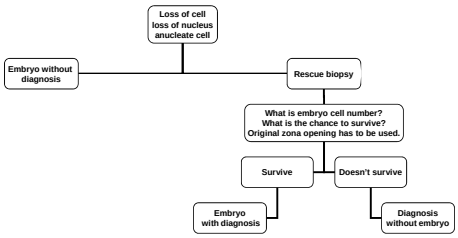
- Purpose:
- to obtain good nuclear quality
- no cell should be lost
- each cell should be informative
- Cummulus cells should be removed properly prior to biopsy as those can contaminate the slide with maternal cells and lead to inaccurate results
- Blastomeres are observed under a stereomicroscope during spreading to ensure a nucleus is present

Spreading of lysed cell

- Lysed cell can be used for analysis, but diagnosis shouldn't be based on lysed cell only
- Lysed cell spreading should be done as soon as possible



No nucleus found after spreading



Recommendations

- There are many ways how to do biopsy and spreading. You need to select the one which works for you and best suits your requirements.
- Set the rules in advance and follow them. Nonstandard processes lead to nonstandard results.
- Documentation helps to keep high quality, localize flaws and evaluate method results.

Thank You for Your Attention