

CHROMATIN QUALITY AS A CRUCIAL FACTOR FOR THE SUCCESS OF FLUORESCENT *IN SITU* HYBRIDIZATION ANALYSES OF UNFERTILIZED OOCYTES, POLAR BODIES AND ARRESTED ZYGOTES

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ABSTRACT

Material that is supernumerary or unsuitable for *in vitro* fertilization (IVF) procedures is used for basic and for IVF-related research. Despite the disadvantages of such cells, they have contributed much to our understanding of the mechanisms and prevalence of different abnormalities.

Fifty-four human unfertilized oocytes, 34 arrested bipronuclear zygotes and 15 polar bodies were fixed for analysis on the third day after *in vitro* insemination and were subjected to fluorescent *in situ* hybridization (FISH) with probes for chromosomes 18, 21, X and Y (centromere for 18, X, Y and locus-specific for 21). The aim of the study was the comparison of FISH efficiency in differently condensed chromatin.

The success of FISH analysis was over 60% of analyzed cells and it was dependent on the chromatin changes (condensation and/or fragmentation) during the culture period before cell fixation. Chromatin ageing was the crucial factor for the reduced success

of FISH in both oocyte chromosomes (60.0%) and pronuclei (61.76%). The chromatin of second polar bodies (PBII), and premature chromosome condensation (PCC) of the sperm chromatin in oocytes was more suitable for FISH analysis (FISH success 75.0% in PBII and 64.29% in PCC) with both centromere and locus-specific probes.

These results revealed the significance of early signs of *in vitro* cell ageing for the success of FISH analysis and for the interpretation of results in case of analysis of unfertilized human ova, polar bodies and arrested zygotes.

Keywords: Chromatin ageing, Fluorescent *in situ* hybridization (FISH), Oocyte, Polar body, Zygote

INTRODUCTION

Genetic investigations of human oocytes are an important source of knowledge about chromosomal aberrations of the embryo, which is the major factor determining the success of assisted reproductive techniques. Female meiosis is a major cause for aneuploidy *via* two mechanisms: non division of bivalents during the first division and predivision of chromatids during the second division [1]. These meiotic errors can be revealed by genetic analysis of first and second polar bodies (PBI, PBII), respectively.

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Even in the absence of a male infertility factor, some human oocytes do not undergo fusion with sperm cells and remain unfertilized. Anomalies of human fertilization, such as premature chromosome condensation (PCC) and polyspermy, are attributed to asynchrony between meiotic chromosomal and cytoplasmic maturity of the oocyte. The PCC of the sperm chromatin is due to preserved activity of maturation promoting factor in the ooplasm [2,3]. This status is irreversible and no pronuclei can be formed. Ooplasm immaturity is related to unreliable blockage of polyspermy because of ineffective cortical reaction during fertilization [4].

Anomalies in pronuclear formation are associated with poor prognosis for the success in *in vitro* fertilization and embryo transfer (IVF-ET) practice. An abnormal number of pronuclei indicates abnormal ploid status of the embryo and transfer of mono-pronuclear or three-pronuclear zygotes is avoided [5,6]. Transfer of bi-pronuclear (2PN) zygotes on the third day after insemination is controversial because typically developing embryos at this time are at the 4-cell stage. The delayed 2PN stage can be caused, in some cases, by fluctuations of normal fertilization but most frequently is a sign of fertilization failure [7,8].

Preimplantation genetic analyses of oocytes, polar bodies (PBs) and zygotes were recently performed in order to reveal their chromosomal status and development potential. Despite the numerous studies, the significance of eventual correlation between chromosomal status of such cells and their restricted developmental potential is a question that still remains open [9-14]. For PBs and blastomeres, genetic analyses can also be used for preimplantation screening in assisted reproductive programs in order to prevent the transfer of chromosomally abnormal embryos.

The selection of an appropriate method for the preimplantation genetic analysis of single cells depends on the purpose of investigation and the characteristics of the chromatin. Classical karyotyping of the oocyte metaphase chromosomal set has long been used [15-17]. However, it is technically difficult, time-consuming and dependent on good chromosome spreading. The high degree of chromosome condensation in the oocyte and PBI is a known phenomenon and the chromosome identification using banding

technique is unreliable [18]. Classical karyotyping of zygotes and blastomeres is possible only after cytostatic pretreatment [9,19,20], which makes the method even more time-consuming and unsuitable for diagnosis before embryo transfer. Interphase chromatin of PBII, pronuclei and blastomeres and metaphase chromosomes of oocytes and PBI can be analyzed by molecular cytogenetic methods [21-25].

Fluorescent *in situ* hybridization (FISH) is currently accepted as an appropriate technique for preimplantation genetic analyses [26-28]. It is fast, uncomplicated and suitable for assay of metaphase and interphase chromatin. Fluorescent *in situ* hybridization is presumed to overcome the difficulty of chromosome spreading and, for that reason, to be appropriate for unfertilized oocytes and polar bodies. For the interphase chromatin, FISH allows analysis without cytostatic pretreatment. Up to 12 chromosomes have been detected in two or three rounds of hybridization on the same cell using sets of directly labeled fluorescent probes [12,29,30]. In clinical applications, biopsy and fixation of polar bodies or blastomeres is performed immediately after oocyte retrieval, fertilization or embryo formation. In these cases, FISH is the most appropriate and efficient technique because of the uncomplicated and time-saving procedure that uses commercially available directly labeled probes for all human chromosomes.

Material that is supernumerary or unsuitable for IVF procedures is useful in basic and IVF-related research. Despite the disadvantages of these cells, the results have contributed much to our understanding of the mechanisms and prevalence of different abnormalities. Human oocytes and preimplantation embryos with poor quality or excessive number are used for analysis. The changes associated with *in vitro* cell ageing started at this time and their first manifestation is chromatin condensation and/or fragmentation. Little is known about the potential impact of these processes on performance of FISH [31,32]. The present study aimed to compare the efficiency and success of FISH in human unfertilized oocytes, arrested zygotes and polar bodies which differ in chromatin condensation and quality.

MATERIALS AND METHODS

Fifty-eight patients aged 28-38 years (mean 32.2) were undergoing IVF-ET procedure due to infertility of different etiology. The ovarian stimulation was applied according to previously described standard protocols [31]. The cells were obtained after standard IVF-ET procedure [33,34] at the Infertility Treatment Program "Technobios", Medical University, Sofia, Bulgaria. A hundred and three cells were included in the present study: 54 unfertilized human oocytes, 34 arrested human zygotes and 15 polar bodies. Informed written consent was obtained from the patients before their surplus material was used for scientific studies. The study was approved by the Ethical Committee of the Medical University, Sofia, Bulgaria and complied with the ethical rules and laws of the European Union.

Oocytes and 2PN zygotes were considered to be unfertilized if they lacked pronuclei, and arrested if they contained two pronuclei but no sign of cleavage on the day of the embryo transfer, *i.e.*, after 48-52 hours of *in vitro* incubation after their *in vitro* insemination. Cell-fixation procedure used the following steps: 1) *zona pellucida* removal by Tyrode's solution (pH 2.5) treatment, observed with a stereomicroscope; polar bodies were fixed together with the unfertilized oocytes and arrested zygotes; 2) hypotonic incubation (1% sodium citrate with 5% BSA) for 20 min. at room temperature; 3) cells were placed onto acetone-precleaned slides and a cold fixative (1:3 acetic acid:methanol) was applied. Disappearance of the cytoplasm during fixation was controlled using phase contrast microscopy.

Oocyte metaphases were stained with Giemsa for visualization and evaluation of chromosomes. Only oocytes with good metaphase spread (40 cells) or with PCC of the sperm chromatin beside the female chromosomes (14 cells) were included in the study. The cells were then discolored and frozen for further analysis by FISH. Slides with zygotes and polar bodies were frozen without Giemsa staining.

The chromatin of oocytes, pronuclei and polar bodies was FISH-analyzed for chromosomes 18, 21, X and Y to investigate the ploidy status of the cells. The slides were prepared for FISH by dehydration in ethanol (70, 85 and 100%) and additional drying and fixation at 60°C for 1 hour. Fluorescent *in situ*

hybridization was performed in two cycles using a centromere- and a locus-specific probe at each step. At the first cycle of hybridization, probes for chromosomes 18 (centromere-specific, CEP 18 Spectrum Green; Vysis, Stuttgart, Germany) and 21 (locus-specific, LSI 21 Spectrum Orange; Vysis) were used. The probes and the chromatin were simultaneously denatured at 73°C for 7 min. Hybridization was performed in a humid chamber for 5 hours at 37°C. The chromatin was counterstained by DAPI (4',6-diamidino-2-phenylindole). Between the two FISH steps, the results were registered and the slides were then washed in dH₂O for 10 seconds at 71°C, dehydrated in ethanol series and warmed to 60°C again. At the second cycle of hybridization, probes for chromosomes X and Y (centromere-specific, FITC-labeled for chromosome X and classical satellite probe, Texas Red Labeled for chromosome Y; Q-Biogene, Heidelberg, Germany) were applied. The procedures of denaturation, hybridization and counterstaining were the same as at the first step. Results of FISH were observed by fluorescent microscopy (BX60; Olympus, Hamburg, Germany), digitally registered (VC45; Olympus) and analyzed using ISIS software (Metasystems, Altlussheim, Germany).

RESULTS

The visible quality of chromatin varied widely between analyzed cells. In unfertilized oocytes with good metaphase spread, chromosomes had a normal appearance in some cells but were more condensed or fragmented in others. When PCC was present, paternal chromosomes were slightly condensed (with prometaphase appearance), while maternal ones were highly condensed and degenerative/fragmented. This latter picture was also observed in the chromosomes of PBI. Chromatin quality varied in the pronuclei of arrested zygotes: apparently normal interphase or slightly to highly condensed as if undergoing apoptosis. The chromatin of PBII tended to resemble the chromatin of the respective arrested zygote.

The success of FISH analyses in unfertilized oocytes correlated with chromosomal morphology. Successful hybridization was detected in 24 of 40 analyzed metaphases (60.0%). Both centromere- and locus-specific signals were detected in nine of

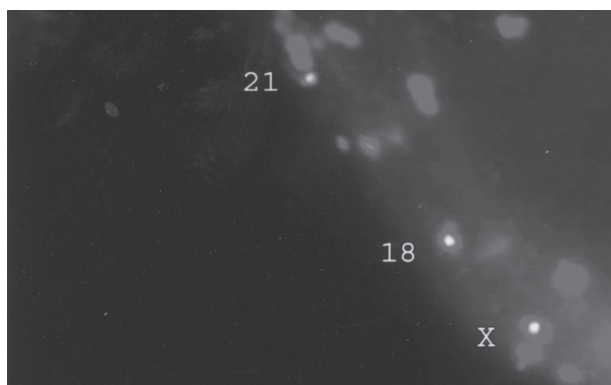


Figure 1. Positive FISH reaction in a DAPI stained oocyte metaphase with centromere (for 18 and X chromosomes) and locus-specific probes (for 21 chromosome). The images of two hybridization rounds were digitally merged. Original magnification 1000x.

these 24 cells (37.50%) (see Figure 1). All these oocytes had been shown to have good metaphase plates by Giemsa staining. The other FISH positive metaphases (15 of 24 cells, 62.50%) displayed only the centromere signals. These oocytes displayed two types of progressive chromatin degeneration as extremely condensed (Figure 2) or “fuzzy” chromosomes (Figures 3 and 4). The 16 oocytes with no FISH signals had more pronounced chromatin degeneration.

Fourteen unfertilized oocytes contained prematurely condensed sperm chromosomes (PCC) with prophase or prometaphase appearance beside the oocyte plate (Figure 3). Maternal chromosomes

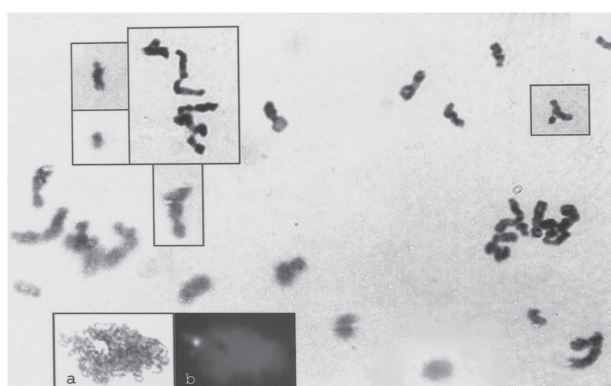


Figure 3. A combined image of the chromosomal set displaying three types of chromosomes: a group with a fuzzy appearance, a group with a prometaphase view and a PCC group (the insets “a” and “b”). The PCC is Giemsa-stained at the inset “a” and DAPI stained image displaying single X signal (FITC-labelled) is at the inset “b”. Original magnification 1000x.

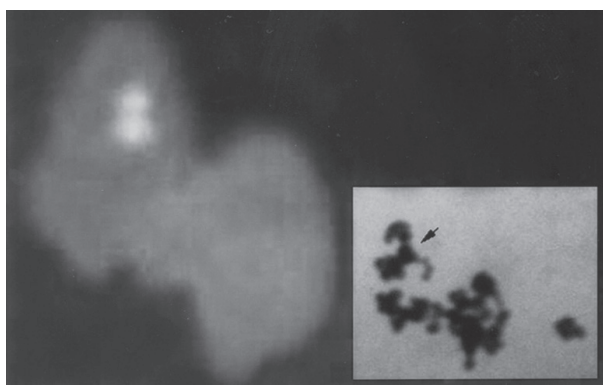


Figure 2. Localization of 18 chromosome in an oocyte with extremely clustered and fragmented chromosomes (DAPI counterstaining). The signal looks double because of the two sister chromatids of chromosomes. At the inset on the right is the image of Giemsa-stained metaphase. Original magnification 1000x.

were visibly degenerated and displayed no signals. However, in nine of these cells (64.29%), the FISH reaction was positive in the group of sperm chromosomes (Figures 3 and 4).

The 34 analyzed zygotes displayed two pronuclei on the day of embryo transfer. Their chromatin condensation varied between the cells and between the pronuclei in the same cell. In 13 zygotes (38.24%) no fluorescent signals were detected. The negative FISH reaction was associated with highly condensed chromatin. In the other 21 zygotes (61.76%), the centromere probes labeled at least one of the pronuclei (Figure 5).

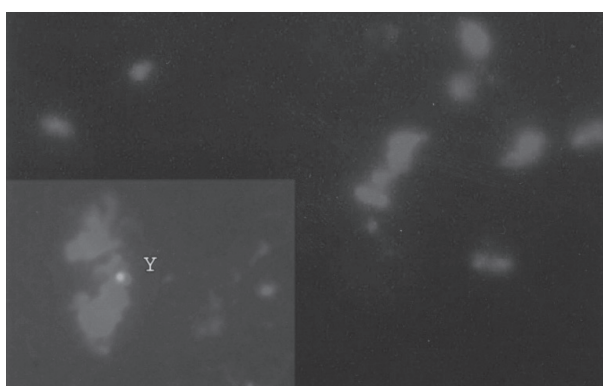


Figure 4. Part of a degenerative oocyte metaphase plate. The PCC group (DAPI counterstaining) at the inset displayed positive FISH reaction for Y chromosome. Original magnification 1000x.

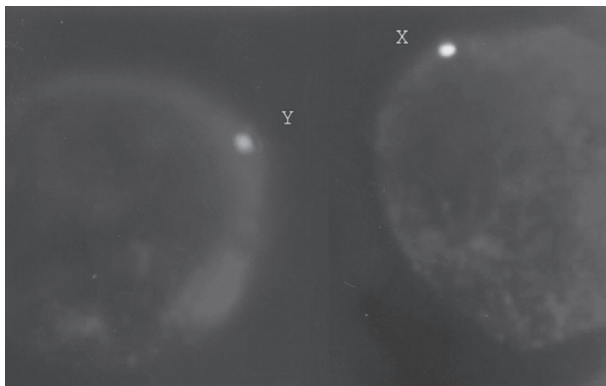


Figure 5. Positive FISH reaction for chromosomes X and Y in 2PN zygote. The chromatin was DAPI counterstained. Original magnification 1000x.

Of the 15 polar bodies, seven were obtained from unfertilized oocytes (PBI) and eight from zygotes, and were regarded to be PBII because of their interphase chromatin appearance. The PBIf had highly condensed, visibly degenerated chromosomes. Hybridization success was low: only three of seven PBI (42.86%) showed centromere signals (Figure 6) and none reacted with the locus-specific probe. In six of eight PBII (75.0%, Figure 7), the chromatin was better preserved and positive FISH signals for centromere- and locus-specific probes were obtained.



Figure 6. Degenerated chromosomes of a first polar body. The FISH reaction was successful for centromere probe for 18 chromosome and negative for chromosome 21. Original magnification 1000x.

DISCUSSION

The FISH analyses of human unfertilized oocytes and arrested zygotes revealed some disadvantages related to chromatin changes before fixation. Hybridization efficiency varied between the cells and between different chromatin groups in the same cell. The results were dependent on the type of fluorescent probe (centromere- or locus-specific) in the same chromatin. The observed differences correlated with chromatin condensation and fragmentation: results of FISH were informative when the chromatin was well preserved (Figures 1 and 7 for both types of probes and in PCC in Figures 3 and 4). Fluorescent *in situ* hybridization was less informative when chromatin quality was poor (Figures 2 and 6).

In arrested 2PN zygotes, FISH efficiency was different and correlated with chromatin morphology. In pronuclei with visibly normal interphase or prometaphase chromatin, hybridization was successful: in slightly condensed pronuclei, centromere signals were registered (shown in Figure 5); in pronuclei with highly condensed and/or fragmented chromatin, FISH was unsuccessful. In other words, pronuclear chromatin displayed different levels of condensation and fragmentation and FISH results were dependent on these changes. These chromatin changes during the prolonged culture period could be attributed to cell ageing and death. In the present study, ageing chromatin of 2PN

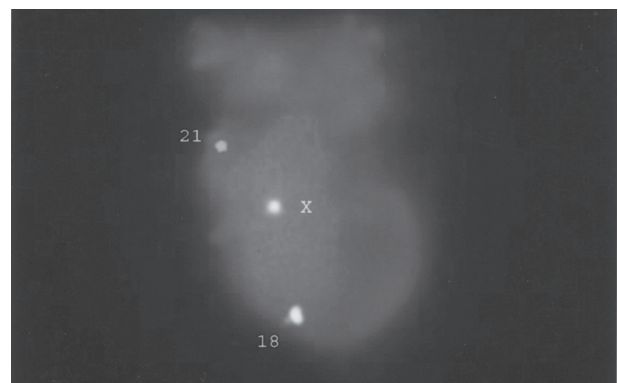


Figure 7. Positive FISH reaction in a DAPI stained chromatin of PBII with centromere- (for 18 and X chromosomes) and locus-specific probes (for 21 chromosome). The images of two hybridization rounds were digitally merged. Original magnification 1000x.

zygotes followed the typical pattern of apoptotic changes previously observed by us in blastomeres of preimplantation embryos with poor quality and low developmental potential [35].

In highly condensed oocyte and PBI metaphase chromosomes, FISH success was low, especially with locus-specific probes. This could be explained by at least two factors, both associated with prolonged culture time. First, the high degree of chromosome condensation, which is a well known characteristic even of normal oocyte metaphase plates [36,37], could advance so much that target sequences would become inaccessible for probes. Second, in some cases, a high degree of chromatin condensation could be a sign of degeneration and be accompanied with actual destruction of target sequences. It should be mentioned that unlike some similar studies using FISH for identification of up to 12 or all chromosomes, where interpretation of hybridization results was not reliable because of signal overlapping/merging [32,38], we applied a small number of probes. This allowed us to conclude that the low success rate of FISH was due to degradation or inaccessibility of target regions in unfertilized human oocytes and PBIs. Although relatively few reports have addressed these processes in gametes, recent *in vivo* and *in vitro* studies indicate that not only polar bodies but also oocytes, similar to somatic cells, can undergo apoptosis [39,40].

The chromatin of PBII and prematurely condensed sperm chromatin (PCC groups in oocytes) was more suitable for FISH analysis with both centromere- and locus-specific probes. This could be explained with their later formation as chromatin groups in cells and, hence, shorter culture time.

The condensation and fragmentation of the chromatin are the first signs of cell ageing processes ahead of fragmentation of the nuclei and cytoplasm during apoptotic cell death [41,42]. Chromatin changes were related to the period of culture before cell fixation and FISH results were dependent on these changes. This was shown in cases of different hybridization success in differently condensed/ fragmented chromatin groups in the same analyzed cell, positive FISH reaction in PCC combined by negative FISH reaction in the oocyte metaphase plate. Unlike the oocyte chromosomes,

in prematurely condensed sperm chromatin (PCC) the visible signs of ageing were not advanced. We could suppose that DNA fragmentation was also more pronounced in the oocyte plate than in the later formed PCC chromosomes. Another factor could be the different level of chromatin condensation of these two groups influencing the accessibility of the target regions to the probes. The difference in FISH success between PBI and PBII could be explained in the same way: At the moment of cell fixation, chromosome condensation and fragmentation were more advanced in PBI than in the later formed PBII.

The observed difference in hybridization success between centromere- and locus-specific reaction suggests that the target sequences of locus-specific probes are more sensitive to degeneration changes. This could be due to the fact that locus-specific probes detect unique DNA sequences, while centromere probes bind to DNA repeats.

Our results concerning polar bodies are of interest with regard to preimplantation genetic screening. Polar bodies are regarded as a source of information for maternal meiotic errors but these cells are normally destined for destruction. Since chromatin degradation begins early through programmed cell death, FISH failure due to chromosome fragmentation could be misinterpreted as aneuploidy. This is especially true for the PBI, which is formed at an early stage.

We found that FISH success correlated with the presence of chromatin changes during the culture of human oocytes, polar bodies and bipronuclear zygotes. This emphasizes the significance of early signs of *in vitro* cell ageing for successful FISH analysis and for the interpretation of results in analysis of unfertilized human ova, polar bodies and arrested zygotes.

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REFERENCES

1. Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* 2001; 2(4): 280-291.
2. Schmiady H, Sperling K, Kentenich H, Stauber M. Prematurely condensed human sperm chromosomes after in vitro fertilization (IVF). *Hum Genet* 1986; 74(4): 441-443.
3. Plachot M, Crozet N. Fertilization abnormalities in human in-vitro fertilization. *Hum Reprod* 1992; 7(Suppl 1): 89-94.
4. van der Ven HH, Al-Hasani S, Diedrich K, Hamerich U, Lehmann F, Krebs D. Polyspermy in in vitro fertilization of human oocytes: frequency and possible causes. *Ann NY Acad Sci* 1985; 442: 88-95.
5. Rosenbusch BE. Mechanisms giving rise to triploid zygotes during assisted reproduction. *Fertil Steril* 2008; 90(1): 49-55.
6. Noyes N, Fino ME, Krey L, McCaffrey C, Adler A, Grifo J. Embryo biopsy: the fate of abnormal pronuclear embryos. *Reprod Biomed Online* 2008; 17(6): 782-788.
7. Schmiady H, Kentenich H. Cytological studies of human zygotes exhibiting developmental arrest. *Hum Reprod* 1993; 8(5): 744-751.
8. Lechniak D, Pers-Kamczyc E, Pawlak P. Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos. *Reprod Biol* 2008; 8(1): 23-42.
9. Rosenbusch B, Glaeser B, Brucker C, Schneider M. Endoreduplication of the hyperhaploid maternal complement and abnormal pronuclear formation in a human zygote obtained after intracytoplasmic sperm injection. *Ann Genet* 2002; 45(3): 157-159.
10. Rosenbusch B, Schneider M, Gläser B, Brucker C. Cytogenetic analysis of giant oocytes and zygotes to assess their relevance for the development of digynic triploidy. *Hum Reprod* 2002; 17(9): 2388-2393.
11. Benkhalifa M, Kahraman S, Caserta D, Domez E, Qumsiyeh MB. Morphological and cytogenetic analysis of intact oocytes and blocked zygotes. *Prenat Diagn* 2003; 23(5): 397-404.
12. Pujol A, Boiso I, Benet J, Veiga A, Durban M, Campillo M, Egozcue J, Navarro J. Analysis of nine chromosome probes in first polar bodies and metaphase II oocytes for the detection of aneuploidies. *Eur J Hum Genet* 2003; 11(4): 325-336.
13. Otsu E, Sato A, Nagaki M, Araki Y, Utsunomiya T. Developmental potential and chromosomal constitution of embryos derived from larger single pronuclei of human zygotes used in in vitro fertilization. *Fertil Steril* 2004; 81(3): 723-724.
14. Reichman DE, Jackson KV, Racowsky C. Incidence and development of zygotes exhibiting abnormal pronuclear disposition after identification of two pronuclei at the fertilization check. *Fertil Steril* 2009; May 22 [Epub ahead of print].
15. Tarkowski AK. An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics* 1966; 5: 394-400.
16. Kamiguchi Y, Funaki K, Mikamo E. A new technique for chromosome study of murine oocytes. *Proc Jpn Acad* 1976; 52: 316-319.
17. Delimitreva S, Markova M, Zhivkova R. Optimal metaphase plate preparation in mouse oocytes requires specific conditions for spindle disintegration. *Comptes Rendus de l'Academie Bulgare des Sciences* 1999; 52(7-8): 105-108.
18. Martini E, Flaherty SP, Swann NJ, Payne D, Matthews CD. Analysis of unfertilized oocytes subjected to intracytoplasmic sperm injection using two rounds of fluorescence in-situ hybridization and probes to five chromosomes. *Hum Reprod* 1997; 12(9): 2011-2018.
19. Pellestor F. The cytogenetic analysis of human zygotes and preimplantation embryos. *Hum Reprod Update* 1995; 1(6): 581-585.
20. Pellestor F, Girardet A, Andréo B, Arnal F, Humeau C. Relationship between morphology and chromosomal constitution in human preimplantation embryo. *Mol Reprod Dev* 1995; 39(2): 141-146.
21. Wells D, Sherlock JK, Handyside AH, Delhanty JD. Detailed chromosomal and molecular genetic analysis of single cells by whole genome amplification and comparative genomic hybridization. *Nucleic Acids Res* 1999; 27(4): 1214-1218.
22. Paulasova P, Andréo B, Diblík J, Macek M, Pellestor F. The peptide nucleic acids as probes for chromosomal analysis: application to human oocytes, polar bodies and preimplantation embryos. *Mol Hum Reprod* 2004; 10(6): 467-472.

23. Gutiérrez-Mateo C, Benet J, Wells D, Colls P, Bermúdez MG, Sánchez-García JF, Egozcue J, Navarro J, Munné S. Aneuploidy study of human oocytes first polar body comparative genomic hybridization and metaphase II fluorescence in situ hybridization analysis. *Hum Reprod* 2004; 19(12): 2859-2868.
24. Fragouli E, Wells D, Thornhill A, Serhal P, Faed MJ, Harper JC, Delhanty JD. Comparative genomic hybridization analysis of human oocytes and polar bodies. *Hum Reprod* 2006; 21(9): 2319-2328.
25. Daphnis DD, Fragouli E, Economou K, Jerkovic S, Craft IL, Delhanty JD, Harper JC. Analysis of the evolution of chromosome abnormalities in human embryos from Day 3 to 5 using CGH and FISH. *Mol Hum Reprod* 2008; 14(2): 117-125.
26. Kuliev A, Verlinsky Y. Meiotic and mitotic nondisjunction: lessons from preimplantation genetic diagnosis. *Hum Reprod Update* 2004; 10(5): 401-407.
27. Harper JC, Pergament E, Delhanty JD. Genetics of gametes and embryos. *Eur J Obstet Gynecol Reprod Biol* 2004; 115(Suppl 1): S80-S84.
28. Sermon K, Van Steirteghem A, Liebaers I. Preimplantation genetic diagnosis. *Lancet* 2004; 363(9421): 1633-1641.
29. Baart EB, Martini E, Van Opstal D. Screening for aneuploidies of ten different chromosomes in two rounds of FISH: a short and reliable protocol. *Prenat Diagn* 2004; 24(12): 955-91.
30. Colls P, Goodall N, Zheng X, Munné S. Increased efficiency of preimplantation genetic diagnosis for aneuploidy by testing 12 chromosomes. *Reprod Biomed Online* 2009; 19(4): 532-538.
31. Zhivkova RS, Delimitreva SM, Toncheva DI, Vatev IT. Analysis of human unfertilized oocytes and pronuclear zygotes-correlation between chromosome/ chromatin status and patient-related factors. *Eur J Obstet Gynecol Reprod Biol* 2007; 130(1): 73-83.
32. Uher P, Baborova P, Kralickova M, Zech MH, Verlinsky Y, Zech NH. Non-informative results and monosomies in PGD: the importance of a third round of re-hybridization. *Reprod Biomed Online* 2009; 19(4): 539-546.
33. Vatev I. Human in vitro fertilization and embryo transfer program at the Medical Academy, Sofia, Bulgaria. *J In Vitro Fert Embryo Transfer* 1988; 5(1): 48-49.
34. Vatev I., Karagyzov I, Istatkov M, Srebrevia M. Successful application of the fallopian sperm perfusion method and intracytoplasmic sperm injection (ICSI) technique for the treatment of human infertility. *Balkan J Med Genet* 1998; 3(1): 115-119.
35. Delimitreva SM, Zhivkova RS, Vatev IT, Toncheva DI. Chromosomal disorders and nuclear and cell destruction in cleaving human embryos. *Int J Dev Biol* 2005; 49(4): 409-416.
36. Martin RH, Mahadevan MM, Taylor PJ, Hildebrand K, Long-Simpson L, Peterson D, Yamamoto J, Fleetham J. Chromosomal analysis of unfertilized human oocytes. *J Reprod Fertil* 1986; 78(2): 673-678.
37. Pellestor F, Anahory T, Hamamah S. The chromosomal analysis of human oocytes. An overview of established procedures. *Hum Reprod Update* 2005; 11(1): 15-32.
38. Oliver-Bonet M, Benet J, Martin RH. Studying meiosis: a review of FISH and M-FISH techniques used in the analysis of meiotic processes in humans. *Cytogenet Genome Res* 2006; 114(3-4): 312-318.
39. Perez GI, Acton BM, Jurisicova A, Perkins GA, White A, Brown J, Trbovich AM, Kim MR, Fissore R, Xu J, Ahmady A, D'Estaing SG, Li H, Kagawa W, Kurumizaka H, Yokoyama S, Okada H, Mak TW, Ellisman MH, Casper RF, Tilly JL. Genetic variance modifies apoptosis susceptibility in mature oocytes via alterations in DNA repair capacity and mitochondrial ultrastructure. *Cell Death Differ* 2007; 14(3): 524-533.
40. Morita Y, Tilly JL. Oocyte apoptosis: like sand through an hourglass. *Dev Biol* 1999; 213(1): 1-17.
41. Delimitreva SM. The status of the chromatin of human preimplantation embryos with good morphology. *Folia Biol (Praha)* 2002; 48(4): 149-153.
42. Gjorret JO, Knijn HM, Dieleman SJ, Avery B, Larsson LI, Maddox-Hyttel P. Chronology of apoptosis in bovine embryos produced in vivo and in vitro. *Biol Reprod* 2003; 69(4): 1193-1200.