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Morphology and Aneuploidy of *in vitro* Matured (IVM) Human Oocytes

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1. Introduction

In vitro matured (IVM) human oocytes are very sensitive to culturing conditions. There are many external factors influencing their nuclear and cytoplasmic maturation, which can lead to morphologic or genetic abnormalities. Even oocytes themselves can possess some intrinsic abnormalities in biochemical cell cycle regulation which prevent them from reaching maturity *in vivo*. The following chapter will describe different morphological characteristics and their possible connection to aneuploidy, fertilization and embryo development. Also it will cover some aspects of oocyte IVM and describe mechanisms leading to aneuploidy. The second part of the chapter will be devoted to fluorescent *in situ* hybridization (FISH) analysis of IVM oocytes and first polar bodies (PB1) with the emphasis on aneuploidy occurrence in oocytes with prolonged cultivation.

2. Changes in the oocyte during maturation (oogenesis)

2.1 Introduction

Oogenesis includes many mechanisms that enable cytoplasmic and nuclear maturation in exact timing and succession. The first meiotic arrest occurs early in prenatal life when the oocyte proceeds through the first stages of meiosis and stops at the diplotene of the first prophase. This stage is called germinal vesicle stage (GV) and is characterized by a clearly visible large nucleus with nucleolus (Figure 1b). The maintenance of the meiotic arrest involves many complex molecular mechanisms and interactions with cumulus cells that communicate with the oocyte through cell junctions (Brower & Schultz, 1982; Gilchrist et al., 2004; Goud et al., 1998; Motta et al., 1994).

Human oocytes acquire the ability to overcome the meiotic arrest during simultaneous nuclear and cytoplasmic maturation. During nuclear maturation chromatin remodeling enables the transition of the oocyte through the succeeding meiotic phases from the first prophase to the second meiotic metaphase (M II; Figure 1d), where the second meiotic arrest occurs. On the other hand, cytoplasmic maturation involves synthesis of ribonucleic acids (RNA) and proteins, oocyte growth, rearrangement of organelles and cytoskeleton changes (Albertini et al., 2003). Research on mice has shown that nuclear and the cytoplasmic

maturation can be achieved independently. Nevertheless, normal oocyte development can only take its proper course if the two modes are temporarily matched (Eppig, 1996).

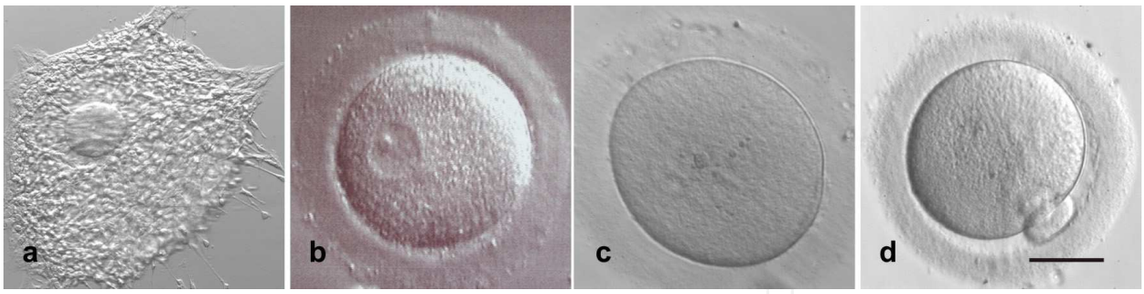


Fig. 1. Stages of oocyte maturation.
Oocyte surrounded by cumulus cells (a), Immature oocyte with visible germinal vesicle (GV) after mechanical and enzymatic removal of cumulus cells (b); immature oocyte in first metaphase (M I) (c); mature oocyte in second metaphase (M II) with clearly visible first polar body (PB1) (d). Magnification: ×100 (a), ×400 (b - d); bar: 150 µm (a), 50 µm (b - d).

2.1.1 Chromatin

Growth of a GV oocyte depends on intensive RNA synthesis which stops as the oocyte reaches its final size (Miyara et al., 2003). Studies including animal and human oocytes have shown that chromatin in GV oocytes of different final sizes is distinctly organized, which affects their maturation competence and later determines the course of embryo development (Combelles et al., 2002; Motlik & Fulka, 1976; Lefevre et al., 1989; Liu et al., 2006; Sun et al., 2004; Zuccotti et al., 1995). Chromatin remodeling includes morphologic changes such as condensation and de-condensation as well as functional changes such as transcription of particular chromatin regions. The major post-translational biochemical events during remodeling include acetylation and phosphorylation of different histones and chromatin methylation (De La Fuente, 2006; Spinaci et al., 2004).

Chromatin condensation is an indirect indicator of the cessation of gene transcription as well as RNA translation into proteins that have been taking place during the growth phase (Albertini et al., 2003). Those oocytes that have reached their final size and at the same time have the majority of chromatin configured in a circle around the nucleolus with some dense chromatin granules scattered across the nucleus, have optimum maturation capacity (Combelles et al., 2002).

The first visible sign of meiosis resumption is a gradual germinal vesicle breakdown (GVBD). The nuclear envelope disintegrates while the chromosomes remain at their position (Motlik & Fulka, 1976). *In vitro*, GVBD starts in less than 6 hours after the removal of the oocyte from the ovary (Combelles et al., 2002), presuming that the oocyte has already reached its final size before the establishment of *in vitro* conditions (Mrazek & Fulka, 2003). The GVBD phase itself lasts for approximately 12 hours (Angell, 1995).

2.1.2 Cytoskeleton

GV oocytes possess a dense sub-cortical microtubular network that is characteristic of interphase cells (Combelles et al., 2002). At the onset of GVBD, microtubules begin to

assemble in the centrosomes (which contain microtubules organizing centers - MTOCs) as small star-like structures called asters, whereas there are no free microtubules in the cytoplasm. Later, the microtubules elongate and asters migrate towards opposite poles forming a barrel-like structure called meiotic spindle. The highly condensed chromosomes attach to the microtubules at their centromeric regions and align in the metaphase equatorial plane between both poles of the meiotic spindle (Battaglia et al., 1996).

In the first anaphase, chromosomes move toward the poles of the meiotic spindle located at the animal pole of the oocyte (Matsuura & Chiba, 2004). In the next phase, namely the first telophase microtubules can be observed as very dense network of threads spreading between the chromatin of the oocyte and its first polar body (PB1).

Actin filaments, also called microfilaments, regulate various dynamic processes during oocyte maturation. Although they are not directly involved in GVBD and meiotic spindle formation, they play a key role in rearrangement of cell organelles and cell polarization (Albertini et al., 2003). They are also responsible for the positioning of the meiotic spindle, movement and separation of chromosomes and extrusion of the PB1 (Kim et al., 1998; Sun & Schatten, 2006).

2.1.3 Cell organelles

Growth of an oocyte is also characterized by redistribution of cell organelles into a sub-cortical region beneath the cell membrane, whereas during the maturation phase they are distributed more centrally (Albertini et al., 2003). The only exceptions are the cortical granules which are translocated from the smooth endoplasmic reticulum towards the periphery of the oocyte by the microfilaments and are distributed immediately beneath the plasma membrane in the fully grown mature oocyte (Sun et al., 2001).

The cytoplasm of the oocyte contains many mitochondria providing energy for all cell processes (Van Blerkom, 2004). Their redistribution and gathering around the nucleus (germinal vesicle) during maturation has been attributed to the action of microtubules (Sun & Schatten, 2006).

The cytoplasm of a mature oocyte contains smooth endoplasmic reticulum and lysosomes, whereas there is almost no rough endoplasmic reticulum or ribosomes since the mature oocyte is quiescent and the protein synthesis does not take place (Sathananthan, 1997; Sathananthan et al., 2006).

2.1.4 Biochemical regulation of the cell cycle

One of the most important factors enabling the continuation of the cell cycle is the M-phase (maturation) promoting factor (MPF), consisting of cyclin B and $p34^{cdc2}$ (Gautier et al,. 1988). In the immature oocyte, it is present in its inactive phosphorylated form (Lohka et al., 1988). The amount of cyclin B and consequently the amount of MPF are changing cyclically during the oocyte growth and are highest just prior to the GVBD. Preceding the meiosis resumption, activation of MPF by dephosphorylation takes place.

A key regulator of oocyte maturation process is a product of *c-mos* proto-oncogene called $p39^{mos}$. It belongs to a family of serine/threonine-specific protein kinases and is sensitive to concentration of Ca^{2+} ions, which is involved in activation or stabilization of MPF (Gebauer

& Richter, 1997; Sagata, 1997). A direct consequence of MPF's action on a variety of different molecules in the cell is GVBD, protein phosphorylation and chromosome condensation (Chian et al., 2003; Smitz et al., 2004; Whitaker, 1996). In the first anaphase, the concentration of MPF abruptly declines due to cyclin B degradation. But in the subsequent first telophase, it increases again as the PB1 is extruded and the now mature oocyte is arrested in the second metaphase without the intervening interphase (Dekel, 1995, 2005).

The stimulatory effects of MPF are counteracted by cyclic adenosine monophosphate (cAMP). It acts so as to attain the cell in the quiescent state by activating protein kinase A (PKA) which in turn hinders $p34^{cdc2}$ dephosphorylation and consequently prevents GVBD. Sufficient cAMP concentration is achieved by purine bases such as hypoxanthine and adenosine which impede phosphodiesterase activity thereby stopping the meiosis (Downs et al., 1989). Both molecules enter the oocyte through cell junctions from cumulus cells surrounding it. When the cell junctions are interrupted in the course of maturation, the level of cAMP drops under a critical level and meiosis can resume (Schultz et al., 1983).

3. *In vitro* maturation (IVM) of human oocytes

3.1 IVM methods

The increasing frequency of infertility in women and men has facilitated the development of assisted reproduction technology (ART). Among ART methods is also the *in vitro* maturation (IVM) method, whereby oocytes are aspirated immature and grown in an incubator in a medium supplied with proper growth factors and in suitable atmosphere until they eventually reach M II stage. It has been shown that 34-82 % of the immature oocytes reach M II stage (Cha & Chian, 1998; Chian & Tan, 2002; Goud et al., 1998; Janssenswillen et al., 1995; Kim et al., 2000; Roberts et al., 2002).

Oocytes can be matured *in vitro* following two main protocols. In the first one, oocytes are acquired by aspiration of antral follicles with the diameter of up to 10 mm without any preceding ovarian hormonal stimulation. This method requires presence of cumulus-oophorus cells surrounding the oocyte during the cultivation period, since they secrete certain biochemical factors responsible for proper oocyte development and maturation (Chian et al., 2004a, 2004b; Schramm & Bavister, 1995; Tan & Child, 2002; Trounson et al., 2001). When using the alternative IVM method, oocytes are aspirated after a short hormonal stimulation, with or without the application of human chorionic gonadotropin (HCG), which increases the efficacy and the speed of their maturation (Smitz et al., 2004). In this protocol, cumulus cells are not essential for the oocyte development (Kim et al., 2000; Chian and Tan, 2002), however maturation is more synchronous in the presence of the cumulus oophorus (Kim et al., 2000) and more oocytes reach the maturity (Goud et al., 1998).

Metabolism of immature oocytes differs from that of mature oocytes or embryos. As a consequence, oocyte's maturation competence is affected by the composition of the cultivation medium (Cekleniak et al., 2001; Chian and Tan, 2002; Christopikou et al., 2010; Downs & Hudson, 2000; Herrick et al., 2006; Kovačič &Vlaisavljević, 2002; Sutton et al., 2003). Furthermore, it has been shown that FSH, which is sometimes used to speed up maturation, increases oocyte aneuploidy rate (Roberts et al., 2005; Xu et al., 2011).

In vitro matured oocytes are capable of normal fertilization and embryo development but the success rate is rather low (De Vos et al., 1999; Kim et al., 2000) and only a few children have been born after IVM (Edirisinghe et al., 1997; Friden et al., 2005; Liu et al., 1997; Liu et al., 2003; Nagy et al., 1996; Vanhoutte et al., 2005). Possible reasons include suboptimal IVM conditions as well as genetic and epigenetic characteristics of oocytes themselves. In many human and animal 6 to 8-cell IVM embryos, genome activation does not take place (Kim et al. 2004; Schramm et al., 2003). This hindered embryo development is thought to be a consequence of disturbed cytoplasmic maturation or asyncronicity between the cytoplasmic and nuclear maturation in the oocyte.

In vitro, oocytes begin their maturation earlier than *in vivo* (Motlik & Fulka, 1976) which is most probably a consequence of their premature extraction from the ovaries (Sanfins et al., 2004). Ovarian follicles contain inhibiting factors that retain the oocyte at proper meiotic phase for adequate time. If the oocyte is extracted from the follicle too soon the growth and developmental phase is shortened which can lead to nuclear and cytoplasmic anomalies (Trounson et al., 1998). Furthermore, the majority of IVM oocytes are unable to maintain M II phase until fertilization but undergo spontaneous transition into first mitotic interphase within the next 24 hours. This could be caused by impeded cell cycle regulation at the level of microtubules dynamics or chromatin phosphorylation (Combelles et al., 2002).

Following IVM many oocytes do not reach M II phase at all or they mature but are unable to develop into normal embryos. Reasons for development stagnation could be manifold, ranging from cytoplasmic immaturity and meiotic spindle formation abnormalities (Combelles et al., 2003; Eichenlaub-Ritter et al., 1988; Miyara et al., 2003; Mrazek & Fulka, 2003; Neal et al., 2002; Pickering et al., 1988) to chromatin fragmentation followed by the appearance of micronuclei (Junk et al., 2002). Also, the *in vitro* cultivation conditions can be sub-optimal (Chian and Tan, 2002; Junk et al., 2002; Trounson et al., 2001) or the intrinsic factors such as abnormal cell cycle control or affected gene regulation can impede proper oocyte development (Combelles et al., 2003; Eichenlaub-Ritter & Peschke, 2002; Kim et al., 2004). For all those reasons, the safe routine clinical use of IVM oocytes is still under debate, since the quality of such oocytes seems low. Some investigations have been done concerning structural characteristics of chromatin and meiotic spindle, however little is actually known about chromosomal abnormalities of IVM oocytes.

3.2 Morphological characteristics of IVM oocytes

The cytoplasm of a good-quality mature (M II) human oocyte is clear and homogenous, its perivitelline space containing the oval-shaped first polar body (PB1) is narrow, and the zona pellucida is colorless (Veeck, 1988). During the development of ART methods, it was observed that embryos originating from *in vivo* matured oocytes develop and implant better than those originating form IVM oocytes (Trounson et al., 1998; Mikkelsen & Lindenberg, 2001). Furthermore, an increased number of IVM embryos contain multinuclear blastomeres (Nogueira et al., 2000).

IVM oocytes show similar morphological characteristics as *in vivo* matured oocytes regarding cytoplasm, PB1 and perivitelline space. About 56 % of IVM oocytes have normal morphology, the rest having one (34 %), two (8-9 %) or all three (1-2 %) characteristics abnormal (Mikkelsen & Lindenberg, 2001).

3.2.1 Cytoplasm

The cytoplasm is normal in about 72-88 % of IVM oocytes (Figure 2a), but it can also be evenly granular (8-13 %, Figure 2b), unevenly granular (4 %, Figure 2d) or it can contain vacuoles (2–3 %, Figure 2c) (Balaban et al., 1998; De Sutter et al., 1996; Van Blerkom & Henry, 1992). The length of the cultivation period as well as hormonal stimulation, patients' age and cultivation medium somewhat change the frequency of each cytoplasmic type (Xia, 1997; Van Blerkom & Davis, 2001).

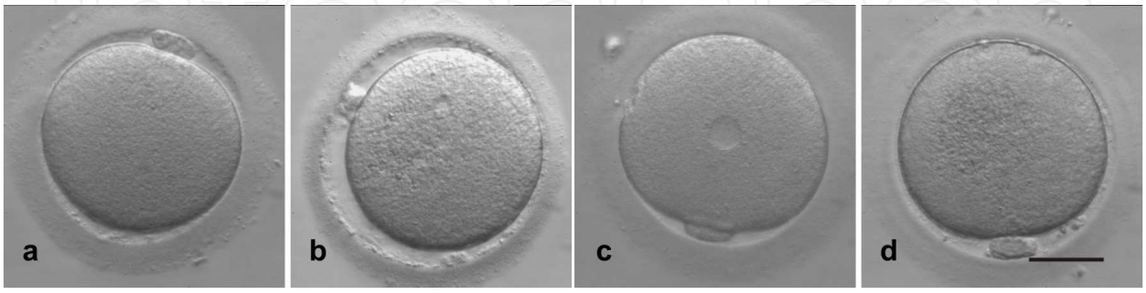


Fig. 2. Cytoplasm morphology of IVM oocytes.
Cytoplasm of a mature oocyte can be normal (a), evenly granular and darker (b), vacuolated (c) or unevenly granular resembling halo (d). Magnificaion: ×400; bar: 50 µm.

The presence of cytoplasmic inclusions is a marker of cellular irregularities which can lead to decreased fertilization as well as decreased number of good-quality embryos. Blastomere division and embryo quality is most strongly affected by cytoplasmic irregularities such as granular or dark cytoplasm and vacuoles (Mikkelsen & Lindenberg, 2001).

In mature oocytes from stimulated ART cycles, seven types of cytoplasmic irregularities were identified that were clearly associated with various fertilization and embryo development problems as well as with chromosomal abnormalities (Van Blerkom, 1990; Van Blerkom & Henry, 1992). The greatest frequency of aneuploid oocytes (32-50 %) has been observed among oocytes with dark or granular cytoplasm, and with clustered organelles such as smooth endoplasmic reticulum (Van Blerkom, 1990; Otsuki et al., 2004).

It seems that vacuoles have little influence on the aneuploidy frequency (4 % of vacuolated oocytes are aneuploid). However, increasing vacuole diameter negatively influences the fertilization rate because vacuoles interfere with cytoskeleton functioning and formation of the meiotic spindle (Ebner et al., 2005; Van Blerkom, 1990).

3.2.2 First polar body (PB1)

The most reliable indicator of nuclear maturity of the oocyte is the extruded first polar body (PB1), whose fragmentation has clearly been linked to decreased fertilization, embryo development, blastulation and implantation (Ebner et al., 1999, 2000, 2006).

PB1 of IVM oocytes shows four major morphological appearances. It can be normal – oval shaped (68-71 %, Figure 3a), fragmented (9 %, Figure 3b), invaginated (7-18 %, Figure 3c) or enlarged (3-6 %, Figure 3d). There is a clear tendency towards increasing frequency of invaginated PB1s in those oocytes that need longer time to attain maturity, suggesting that some intrinsic cellular mechanisms, which are most probably affecting proper meiotic

spindle functioning, prevent normal diakinesis and PB1 extrusion. Further evidence for impaired meiotic spindle functioning in oocytes with invaginated PB1 is only 25 % fertilization rate and embryo arrest at a 2-cell stage (Xia, 1997).

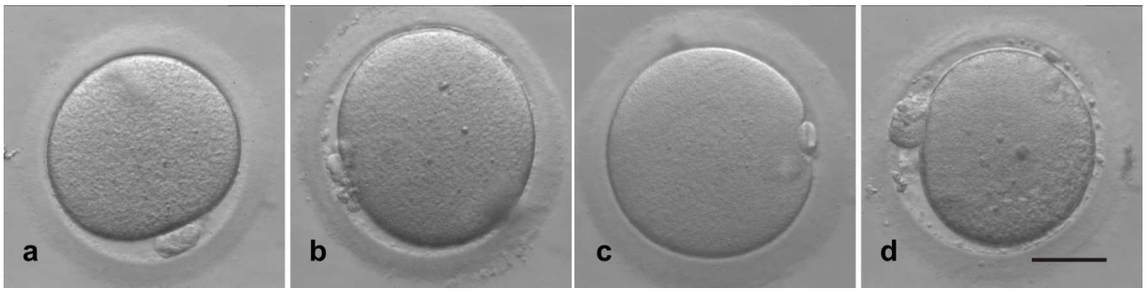


Fig. 3. First polar body (PB1) morphology of IVM oocytes.
PB1 can be normal (a), fragmented (b), invaginated (c) or enlarged (d). Magnificaion: ×400; bar: 50 μm.

Morphology of PB1 strongly depends on the age of the oocyte. Very soon after its extrusion it begins to deteriorate which changes its appearance in just a few hours. Mainly the deterioration is seen as PB1 fragmentation, which implies that attention has to be paid to morphology evaluation timing (Ciotti et al., 2004; Verlinsky et al., 2003). In IVM procedures, PB1s are usually assessed within a few hours after being extruded from the oocyte. Consequently, the frequency of fragmented PB1s (9 %) is a reflection of its actual morphology anomalies. If the incidence of fragmentation is assessed among *in vivo* matured oocytes, the frequency is usually 25-34 % (Ciotti et al., 2004; Verlinsky et al., 2003), which might be a consequence of evaluation timing after the PB1 extrusion, or an aftermath of stimulation protocols, cultivation media or even high concentrations of hormones in the follicular fluid, to which the *in vivo* matured oocytes are exposed (Xia & Younglai, 2000).

3.2.3 Perivitelline space and zona pellucida

Normally perivitelline space is narrow and contains no inclusions or debris. However, in many oocytes from ART cycles there is a substantial amount of inclusions of various sizes between the plasma membrane and zona pellucida. Their origin is not yet completely elucidated, but most probably they are the remnants of cumulus cells' extensions, through which the oocyte has been communicating with its surrounding during the growth phase. It is interesting to note that no debris is present in the GV stage, whereas its amount increases simultaneously with maturation progression, so that around 4 % of M I cells and 34 % of M II cells have some inclusions in their perivitelline space. This phenomenon is most probably completely physiological (Hassan-Ali et al., 1998).

Perivitelline space morphology is normal in 85-87 % of IVM oocytes (Figure 4a). Some have little (3-4 %) or plenty (1-2 %) of debris under zona pellucida (Figures 4b-c), whereas in 4-10 % of oocytes the perivitelline space is enlarged (Figure 4d). The later is created by a premature release of cortical granules (Okada et al., 1986) and there is a tendency of increasing percentage of oocytes with the enlarged perivitelline space in oocytes with longer duration of IVM.

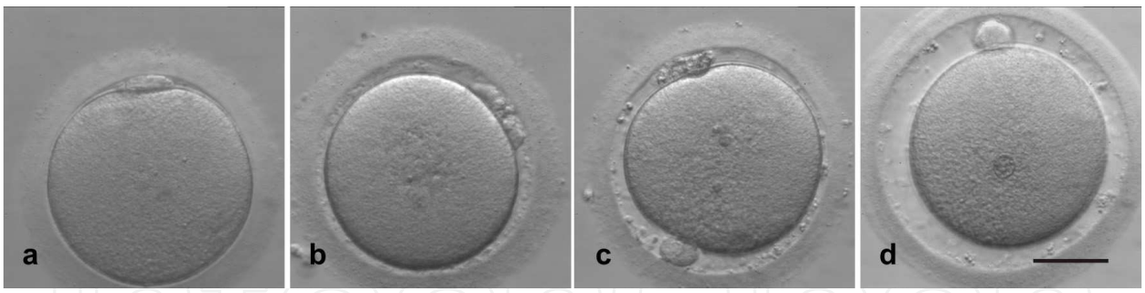


Fig. 4. Perivitelline space morphology of IVM oocytes.
Normal perivitelline space (a) can be easily distinguished from a perivitelline space with little (b) or plenty of debris (c) as well as from the enlarged one (d). Magnificaion: ×400; bar: 50 µm.

Opinions of different researchers on the correlation between oocyte morphology and aneuploidy as well as on implantation and embryo development remain divided due to large discrepancies in ovarian stimulation protocols, oocyte cultivation conditions and embryo assessment criteria (Balaban et al., 1998; Ciotti et al., 2004; De Santis et al., 2005; De Sutter et al., 1996; Ebner et al., 1999, 2000, 2006; Van Blerkom, 1990, 1996; Xia, 1997).

After the introduction of polarization microscopy to human ART methods, it has become possible not only to observe meiotic spindle characteristics and chromosome alignment in live oocytes (Wang et al., 2001) but also to evaluate the integrity of zona pellucida, the outermost barrier between the oocyte and its surrounding. When exposed to polarized light, human zona pellucida shows three-layer architecture defined by different birefringence characteristics (double refraction of light) of each layer. It has been suggested that birefringence is associated with the arrangement of proteins, polysaccharides and glycoproteins within the zona, which change during maturation. The best fertilization rate and embryo development has clearly been linked with those oocytes that possess highly birefringent zona pellucida (reviewed by Montag et al., 2011).

4. Aneuploidy mechanisms during meiosis

4.1 Introduction

Chromosome abnormalities can appear at different stages during oogenesis. Even before the entrance into meiosis, gonadal stem cells divide mitoticaly many times whereby each division represents an opportunity for emergence of gonadal mosaicism (Cozzi et al., 1999). The majority of oocyte aneuploidies originate in the first meiotic division. There are two major mechanisms causing aneuploidies, namely whole chromosome non-disjunction and premature balanced or unbalanced chromatid separation (Cupisti et al., 2003; Delhanty, 2005; Hassold et al., 1995; Kuliev et al., 2003; Kuliev & Verlinsky, 2004; Pellestor, 1991; Pellestor et al., 2002, 2006).

4.2 Chromosome non-disjunction

In case of whole chromosome non-disjunction during anaphase I, a whole tetrad (bivalent; a pair of associated homologous chromosomes, each consisting of two chromatids after

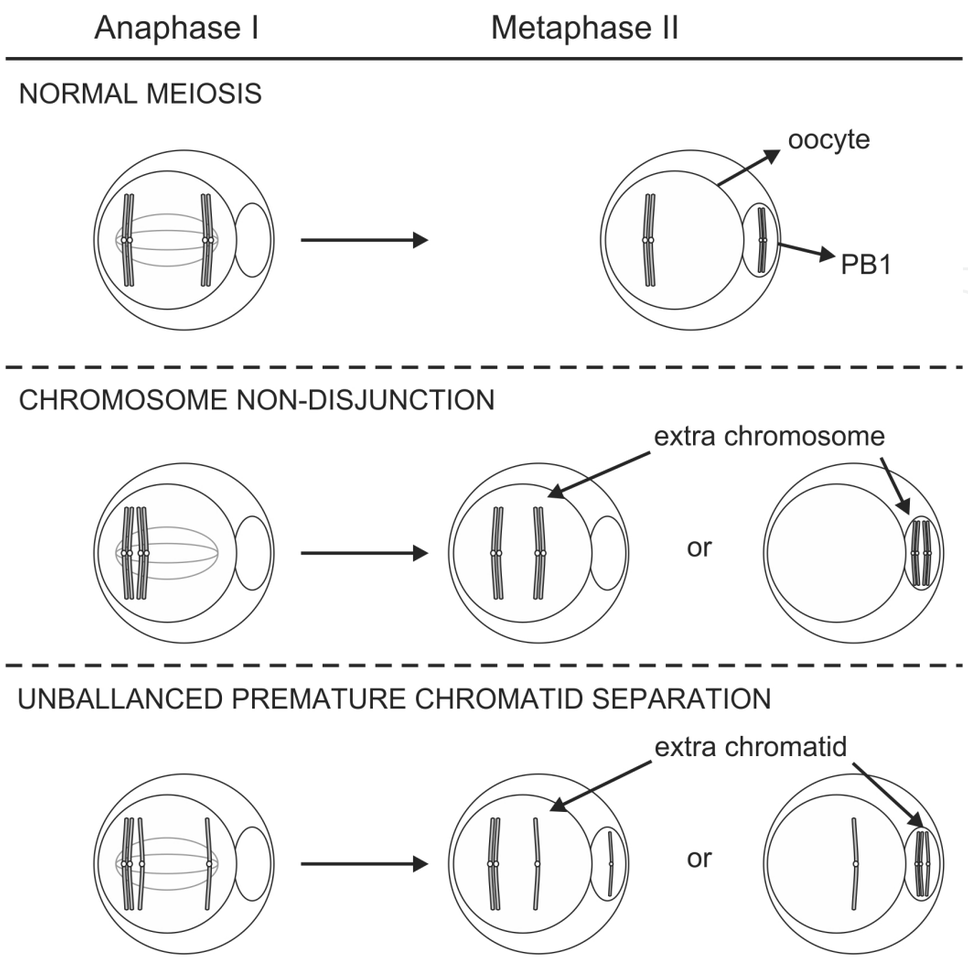


Fig. 5. Aneuploidy types.
Chromatid (FISH signals) arrangement in diakinezis I, anaphase I and metaphase II in case of normal meiosis, chromosome non-disjunction and unballanced premature chromatid separation.

chromosome replication) is moved to one pole of the meiotic spindle instead of the two homologous chromosomes moving one to each pole (Figure 5). Usually the chromatids in the two homologous chromosomes do not remain attached to each other (Angell, 1997). Non-disjunction can affect any chromosome in the cell independently or it can affect all of them at the same time. Upon analyzing an oocyte with fluorescent *in situ* hybridization (FISH) four signals in an oocyte (disomy, Figure 7b) and none in its corresponding PB1 are seen for the affected chromosome. In case of nullisomy no FISH signal for a particular chromosome are present in the oocyte, whereas there are four in its PB1 (Figure 7a).

If the chromosome non-disjunction affects all chromosomes of the oocyte in the same way, the newly formed oocyte is said to be diploid (Figure 7f). All chromosomes remain in the oocyte, whereas in its PB1 there is no genetic material. Non-disjunction is more frequent in oocytes of older women (Angell, 1997; Dailey et al., 1996; Pellestor et al., 2003) and is in cases of chromosomes 15, 16, 18 and 21 sometimes linked to decreased number of chiasmata or changes in recombination sites (Hassold et al., 2000; Lamb et al., 2005).

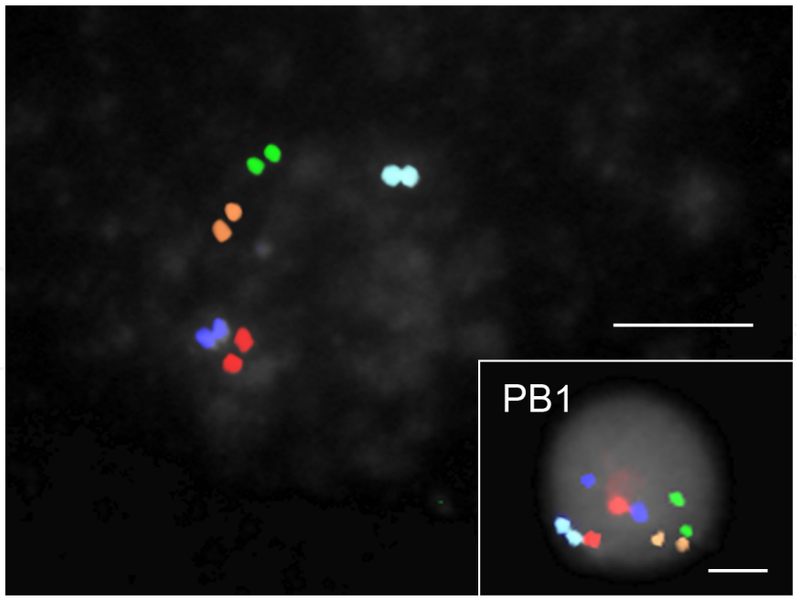


Fig. 6. Normal oocyte and PB1 in IVM oocyte.
In the oocyte and its corresponding PB1 (inset) two signals for each of the chromosomes 13 (red arrows), 16 (light blue arrows), 18 (blue arrows), 21 (green arrows) and 22 (orange arrows) are clearly seen. Each signal represents one chromatid. Magnification ×1000; bar 10 µm.

4.3 Premature chromatid separation

In meiosis I, the most frequent aneuploidy producing mechanism is premature chromatid separation in anaphase I (Fragouli et al., 2011). It is characterized by premature decomposition of cohesins which serve to establish a link between the two sister chromatids after chromosome replication (Michaelis et al., 1997). In case of premature chromatid separation, upon segregation the two free chromatids of one homologous chromosome can be pulled to the same or to different poles of the meiotic spindle. In a situation where both chromatids of one homologous chromosome travel to the same spindle pole, whereas the other homologous chrome travels to the opposite pole, a FISH analysis would show two signals for that particular chromosome in the oocyte as well as in its PB1 (balanced premature chromatid separation). Therefore, a normal chromatid count would be obtained except that in either the oocyte or PB1 the two signals will be separated (homologous chromosome with separated chromatids) whereas in the other the two signals will be close to each other (normal homologous chromosome). This type of premature chromatid separation is more common in oocytes that were exposed to *in vitro* conditions for longer time and its frequency increases from 6 to 53 % within 24-48 hours (Munne et al., 1995b).

In unbalanced premature chromatid separation, chromatids of the affected homologous chromosome travel to opposite poles of the meiotic spindle. If an oocyte contains one whole homologuous chromosome plus one extra chromatid of the other homologuous chromosome, which should normally be expelled from the oocyte into the PB1, FISH analysis would reproduce three signals for that particular chromosome in the oocyte. Such

oocyte is said to be hyperhaploid (Figure 7d) for that specific chromosome, whereas its corresponding BP1 contains only one chromatid and is said to be hypohaploid. In case of one missing chromatid in the oocyte, the oocyte is said to be hypohaploid (Figure 7c) and its PB, which contains three signals is hyperhaploid.

Unbalanced premature chromatid separation can affect any chromosome, but is more frequent in chromosomes of groups D (chromosomes 13 to 15), E (chromosomes 16 to 18) and G (chromosomes 21 and 22) (Pellestor, 1991; Pellestor et al., 2002). The only factor unequivocally linked to increased aneuploidy rate is women age (Dailey et al., 1996; Kuliev et al., 2005; Sandalinas et al., 2002). In younger women (25-34 years of age) the frequency of premature chromatid separation is only 1.5 %, whereas in women aged 40-45 years it increases to more than 24 % (Dailey et al., 1996).

4.4 Aneuploidy of ART oocytes

Most research regarding oocyte aneuploidy in ART cycles has been done on oocytes that failed to be fertilized within 24-48 hours after insemination (Anachory et al., 2003; Angell et al., 1991a, Benkhalifa et al., 2003; Clyde et al., 2003; Cupisti et al., 2003; Kim et al., 2004; Pellestor et al., 2002, 2005, 2006). There are great discrepancies on the aneuploidy rate ranging from 8-54 %, which can be a consequence of different stimulation protocols (Munne et al., 1997), women age (Kuliev et al., 2003, 2005; Pellestor et al., 2003; Sandalinas et al., 2002; Vialard et al., 2006), and number of chromosomes analyzed per oocyte (Gutierrez-Mateo et al., 2004a; Pellestor et al., 2003, 2006; Sandalinas et al., 2002). Studies analyzing all 23 chromosomes revealed very high percentage of abnormal oocytes in unfertilized and *in vitro* matured oocytes (48-57 %, Gutierrez-Mateo et al., 2004a, 2004b) as well as in mature donated oocytes (29-56 % with respect to women age; Sandalinas et al., 2002).

Most frequently chromosomes 13, 15, 16, 21, 22 and X are affected in oocytes (Anahory et al., 2003; Benkhalifa et al., 2003; Clyde et al., 2003; Cupisti et al., 2003; Pellestor et al., 2002; Pujol et al., 2003; Sandalinas et al., 2002), which is similar to most affected chromosomes in embryos that include chromosomes 13, 15, 16, 17, 18, 21, 22, X and Y (Abdelhadi et al., 2003; Munne & Weier, 1996; Munne et al., 2003, 2004), supporting the idea that meiosis I is the main source of aneuploidies in human.

5. Preimplantation Genetic Diagnosis (PGD) on a single cell

Preimplantation genetic diagnosis (PGD) of aneuploidies is usually based on a biopsy of the first or both polar bodies of an oocyte (Durban et al., 1998; Kuliev et al., 2003; Verlinsky et al., 2001), or on a biopsy of 1-2 blastomeres of the created embryo (Gianaroli et al., 1999; Munne et al., 1993, 1995a, 1999). Lately, PGD has also been performed on a few trophectodermal cells of the blastocyst developed from the fertilized oocyte (de Boer et al., 2004). By analyzing oocytes and early embryos, different chromosomal abnormalities including aneuploidies, translocations and mutations can be detected (Durban et al., 2001; Munne et al., 1998a; Verlinsky & Kuliev, 2003; Verlinsky et al., 2004). Selection of chromosomally normal embryos increases implantation rate (Gianaroli et al., 1997, 1999, 2005) as well as decreases the frequency of spontaneous abortions (Munne et al., 2006).

The least invasive PGD method is the analysis of PB1, which is a byproduct of the first meiosis and does not play any significant role in further embryo development. Therefore, making a biopsy on the oocyte and taking its PB1 for aneuploidy analysis does not alter the oocyte's chromosomal status and only minimally affects the oocyte (Verlinsky et al., 1990). However, it provides a very useful indirect means for determining the oocyte's chromosomal status. Supplementing the PB1 analysis with the PB2 analysis, we get a powerful tool for uncovering the majority of aneuploidies derived from the oocyte during meiosis. In this way, ART clinicians can avoid intrauterine transfers of embryos affected by the oocyte's meiotic aneuploidies (Verlinsky et al., 1996, 1997-1998). Information about the oocyte's chromosomal status before fertilization, which we gain from the PB1 analysis, is indirect since PB1 contains exactly the complementary chromosomes / chromatids to the oocyte. Thus, a normal mature oocyte contains one copy of each of the 22 autosomal chromosomes (two chromatids each) and one copy of gonosome X (two chromatids). Exactly the same chromosomal status is found in PB1 of this oocyte. If any chromosomal abnormalities occurred during meiosis I, oocyte could carry an extra chromosome or chromatid, leaving a deficit of a specific chromosome or chromatid in the PB1, or vice versa (Verlinsky & Kuliev, 2000).

5.1 Oocyte, PB1 or blastomere fixation

The most critical step preceding chromosome analysis is PB1 or blastomere fixation. Because in PGD chromosome analysis is frequently done on a single cell, an ideal fixation method should provide as reliable spreading and fixation of chromosomes on the microscope slide as possible. Ideally, no chromosome or chromatid should be lost otherwise artificial aneuploidy would be diagnosed.

Classical fixation method, which uses a solution of methanol and acetic acid followed by air drying of the specimen (Tarkowski, 1966) produces a significant percentage of misdiagnoses due to chromosome or chromatid loss (Sugawara & Mikamo, 1986). Since its invention, it has been modified many times to ensure better reproducibility and reduce genetic material losses (Durban et al., 1998; Wramsby & Liedholm, 1984) or to be adapted for blastomere fixation (Coonen et al., 1994; Dozortsev & McGinnis, 2001). A comparative study has shown that among different fixation methods the one described by Dozortsev and McGinnis (2001) is simple to apply and returns satisfying percentage (83 %) of useful samples (Velilla et al., 2002).

When doing FISH on PB1 or blastomere it is essential to pay much attention to handling of the cell since both are very small and delicate. Therefore, they are easily damaged or even lost during fixation.

The most critical step in specimen preparation for FISH is spreading, during which the cell membrane breaks and chromosomes swim in hypotonic solution on the microscope slide. The amount of the hypotonic solution should be large enough, so that the spreading is sufficient and there is no overlap of chromosomes, enabling clear distinction between different signals (Velilla et al., 2002). At the same time, the spreading should not be too large so that all chromosomes can be clearly seen in a single visual field under large magnification of the microscope (Munne et al., 1996; Munne et al., 1998b). Despite the very precise handling, the percentage of non-applicable preparations due to PB1 loss is still around 12 % (Table 1).

	Total
Analyzed MII oocytes	**192**
Normal oocytes (%)	88 (45,8)
Aneuploid oocytes (%)	88 (45,8)
Lost oocytes during fixation (%)	7 (3,7)
FISH errors (%)	9 (4,7)
Analyzed PB1s	**129**
Oocyte diagnoses confirmed by PB1 diagnosis	102 (79,1)
Lost PB1 (%)	15 (11,6)
FISH errors (%)	8 (6,2)
Non-applicable oocyte preparations with good PB1 preparation (%)	4 (3,1)

Table 1. FISH analysis of chromosomes 13, 16, 18, 21 and 22 (MultiVysion® PB, Vysis) in oocytes and PB1s, which attained maturity after 24-36 hours of IVM in a simple embryo-cultivation medium.

The success of fixation method partially depends on the number of chromosomes tested and the analytical method used. With increasing number of tested chromosomes there is also an increasing probability of signal overlap. This is clearly demonstrated in FISH studies involving 6 to 9 chromosomes, where the percentage of analyzable slides decreased to some 61 % (Anahory et al., 2003; Gutierrez-Mateo et al., 2004a; Pujol et al., 2003). With the use of multicolor-FISH (M-FISH; Clyde et al., 2003), spectral karyotyping (SKY; Marquez et al., 1998; Sandalinas et al., 2002) or centromeric multicolor FISH (cenM-FISH; Gutierrez-Mateo et al., 2005), which enable simultaneous analysis of all 23 chromosomes, the percentage of useful specimen preparations decreases to mere 31-36 %. From the single cell analysis point of view, this low efficiency is inacceptable for clinical use.

5.2 Fluorescent *in situ* hybridization (FISH) and sources of FISH errors

Today, chromosome analysis is mostly done with FISH. It enables staining and detection of whole chromosomes or their parts on the basis of probe fluorescence after attachment to specific DNA sequences. By selecting a whole-chromosome probe, it is possible to identify aneuploidies as well as chromosome or chromatid structural malformations such as breaks, large deletions, translocations, etc. On the other hand, structural aberrations such as breaks, deletions, insertions and translocations cannot be identified with locus specific probes if they lie in the part of the genome not labeled with the probe. When there is a chance for embryo inheriting a known familiar chromosome aberration, a particular probe for a chromosome or locus of interest can be selected.

As already mentioned one of the most frequent FISH errors is a consequence of loosing genetic material during fixation or overspreading of chromosomes on the microscopic slide so that the chromosomes / chromatids cannot be seen in the same visual field. In preliminary studies of IVM oocytes, we had a chance to simultaneously analyze oocytes and their pertaining PB1s (Table 2). Whereas the analysis of the oocytes alone reported 50 % aneuploidy rate, this rate was lower (40.2 %) if pairs of oocyte-PB1 were analyzed. This is because only the diagnoses where the PB1 had exactly complementary chromosome status to the oocyte were considered accurate. The difference between the two approaches gives us an estimate of the occurrence (9.8 %) of FISH errors if only the oocyte is analyzed.

	Total
Analyzed MII oocytes	**176**
% of aneuploid oocytes	50.0
Analyzed MII oocytes with diagnoses confirmed by corresponding PB1s	**102**
% of aneuploid oocytes	40.2
% of FISH errors	**9.8**

Table 2. Estimation of the frequency of FISH errors using oocyte analysis alone compared to analysis of oocyte-PB1 pairs.

Another frequent cause of FISH errors is signal overlap, whereby we cannot discern between one signal and two or more overlapping ones for the same or for different chromosomes. Also there might appear some difficulties when using centromeric probes, since the two signals for sister chromatids may lie so close that it is hard to differentiate between one strong large signal and two weaker ones (usually they appear as two touching dots).

When analyzing chromosomes of the IVM oocytes and PB1s, special attention has to be paid to removing all cumulus oophorus cells surrounding the oocyte, since their genetic material might appear on the FISH specimens and thus cause diagnostic mistakes. Best way to eliminate the cumulus cells is to remove entire zona pellucida, and any attached cells or remnants of the cumulus, by partial enzymatic digestion followed by mechanical pipetting of the oocyte through a very thin pipette.

Because of FISH limitations, there is an increasing use of comparative genomic hybridization (CGH) for single cell analysis. In this method, polymerase chain reaction (PCR) is used to multiply the entire genome of the cell followed by identification of chromosomes or their parts with missing or excess amount of genetic material (Voullaire et al., 1999; Wells et al., 1999). The method is reliable however it does not enable the detection of genetic errors that affect all chromosomes equally (such as diploidy, triploidy, etc.) or balanced premature chromatid separation (Gutierrez-Mateo et al., 2004b). Besides, CGH takes a few days to be carried out, which is inappropriate for ART treatments since the tested embryos cannot be transferred to uterus at the right time or they have to be frozen until the results of genetic analysis are known (Wilton et al., 2003).

5.3 Aneuploidy occurrence in IVM oocytes

There were only few studies regarding chromosome analysis of IVM oocytes done so far, all of them reporting a high incidence of aneuploidy which ranges from 38 to 70 % (Clyde et al., 2003; Gutierrez-Mateo et al., 2004b; Magli et al., 2006; Pujol et al., 2003; Vlaisavljević et al., 2007). Different methods were used to elucidate the oocyte aneuploidy, among which M-FISH (multicolor-fluorescent *in situ* hybridization) and CGH (comparative genomic hybridization) were used to assess all 23 chromosomes and reported 38–48 % aneuploidy rate, that was increasing with the women's' age from 23 % in women 24 - 35 years of age up to 75 % in women older than 36 years (Clyde et al., 2003; Gutierrez-Mateo et al., 2004b). The consequences of the high aneuploidy rate are many abnormal embryos that develop from fertilized IVM oocytes. It was shown that up to 61 % of analyzed IVM embryos contain aneuploid blastomeres (Emery et al., 2005).

There is an increase in aneuploidy rate from 35 % to 49 % with increasing duration of *in vitro* maturation from 24-36 hours (Table 3A). Furthermore, multiple (complex, Figure 7e) aneuploidies are more common if the oocytes are exposed to maturation protocol for longer time (Table 3B). Also the number of affected chromosomes significantly increases with prolonged IVM (Table 3C) (Križančić Bombek L. et al., 2011). Possible reasons for these observations include exposure of oocytes to artificial milieu, lack of some unknown specific signals or growth factors in the cultivation medium, etc. On the contrary, it is also possible that oocytes already containing chromosome aberrations attain maturity later since their chromosomal abnormalities hinder the cell cycle continuation.

A	**24 h**	**36 h**
Analyzed oocyte-PB1 pairs	65	37
Normal oocytes (%)	42 (64.6)	19 (51.4)
Aneuploid oocytes (%)	23 (35.4) [a]	18 (48.6) [a]
B		
Oocytes with single aneuploidy (%)	15 (23.1)	12 (32.4)
Oocytes with double aneuploidy (%)	6 (9.2)	2 (5.4)
Oocytes with multiple aneuploidies (%)	2 (3.1) [b]	4 (10.8) [b]
C		
Analyzed chromosomes	325	185
Aneuploid chromosomes (%)	33 (10.15) [c]	32 (17.3) [c]

Table 3. Aneuploidy frequency of IVM oocytes matured for 24 or 36 hours.
FISH analysis (MultiVysion® PB, Vysis) of chromosomes 13, 16, 18, 21 and 22 in IVM oocytes.
[a] not statistically significant (chi-square test)
[b] No. too small for statistical analysis
[c] $p < 0.05$ (chi-square test)

When considering aneuploidy types, a difference between faster-maturing and slower-maturing oocytes can be observed. There is an increase in occurrence of hyperhaploid chromosomes compared to hypohaploid ones in slower-maturing oocytes (Table 4A) whereas such trend is not observed in oocytes attaining M II phase within 24 hours (Križančić Bombek L. et al., 2011). Similar results regarding oocytes were reported by other authors (Clyde et al., 2003; Gutierrez-Mateo et al., 2004a; Vialard et al., 2006) whereas the opposite situation, namely the excess of hypohaploid chromosomes, was found in studies analyzing PB1s (Kuliev et al. 2003, 2005, 2011) which is logical since the chromosomal status of PB1 is complementary to the oocyte's.

Unbalanced premature chromatid separation is approximately equally frequent in faster-maturing and slower-maturing oocytes (Table 4B) (Križančić Bombek L. et al., 2011) and is thought to be a consequence of negative influence of *in vitro* conditions (Munne et al., 1995b). However it has also been documented in fresh *in vivo* matured oocytes (Sandalinas et al., 2002) confirming the hypothesis that it is one of the mechanisms of aneuploidy emergence (Angell 1991b). On the other hand, whole chromosome non-disjunction is significantly more frequent in oocytes maturing for longer time (Table 4B) suggesting that this second mechanism of aneuploidy emergence is more prone to cultivation conditions.

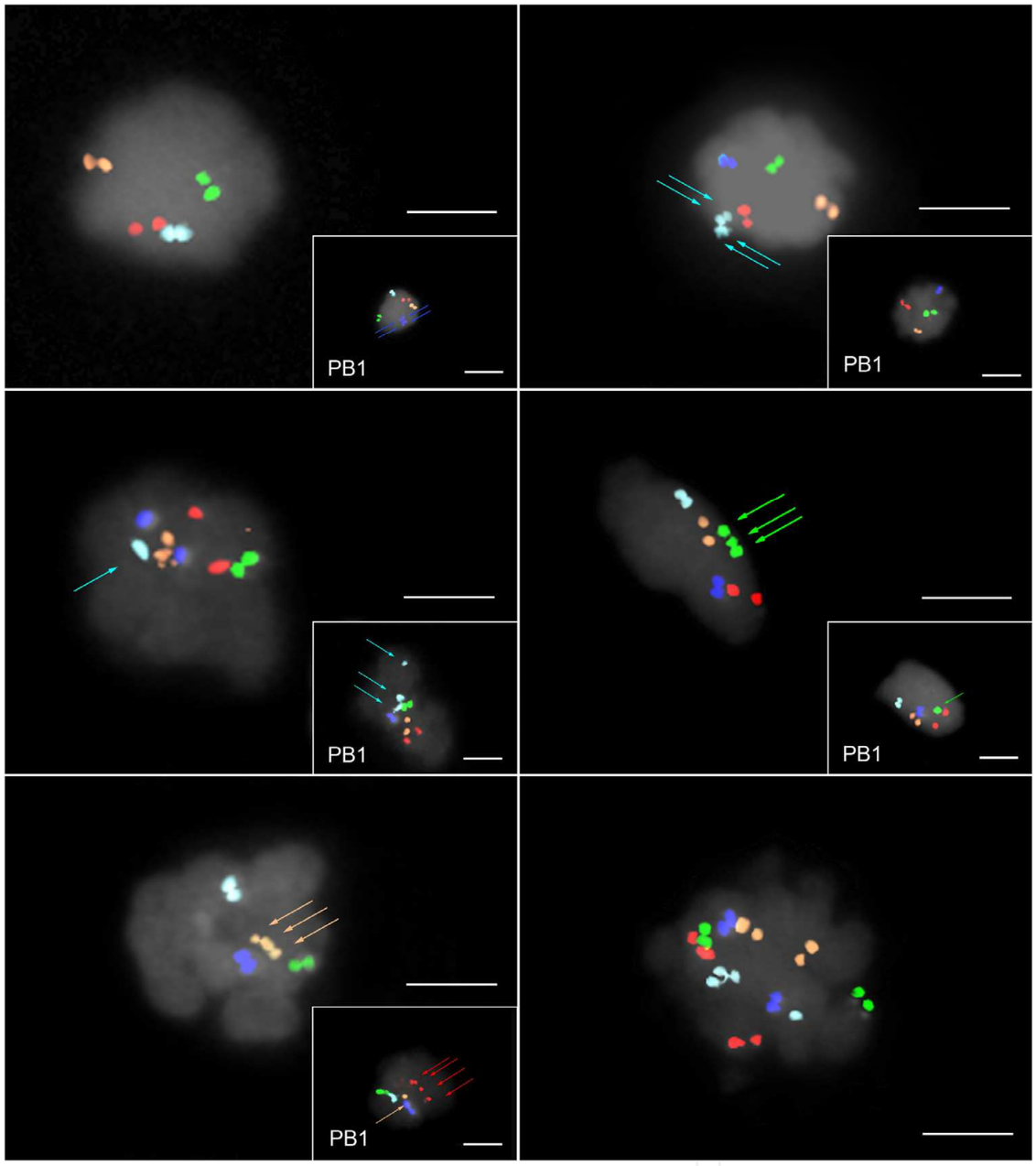


Fig. 7. Aneuploidy types obtained by FISH analysis of chromosomes 13, 16,18, 21 and 22 in IVM oocyte-PB1 pairs. During metaphase I, chromosome non-disjunction may result in nullisomy containing no signal for chromosome 18 (dark blue arrows) in the oocyte and four in its PB1 (a), or in disomy of chromosome 16 (light blue arrows), containing four signals in the oocyte and none in the PB1 (b). On the other hand, unballanced premature chromatid separation may produce hypohaploidy of chromosome 16 (light blue arrows), containing one signal in the oocyte and three in PB1 (c), or hyperhaploidy of chromosome 21 (green arrows), containing three signals in the oocyte and one in the PB1 (d). Furthermore, many IVM oocytes have complex (multiple) aneuploidies of more than just one chromosome (e) or may even be diploid with no genetic material in the PB1 (f). Magnification x1000; bar: 10 µm.

In both aneuploidy mechanisms there is a preference of the genetic material retention in the oocyte instead of its extrusion into the PB1. Consequently there is an excess of hyperhaploid and disomic oocytes over the hypohaploid and nullisomic ones (Table 4C) (Križančić Bombek L. et al., 2011). According to previous analyses of first and second polar bodies (Kuliev et al., 2003, 2005), the existence of an unknown intrinsic mechanism has been proposed that prevents genetic material to be lost from the oocyte during meiosis.

Frequencies and aneuploidy types of individual chromosomes differ, which is influenced by the women age (Benadiva et al., 1996; Petersen & Mikkelsen, 2000; Weier et al., 2005) as well as with the sensitivity of chromosomes themselves (Hassold et al., 2000; Li et al., 2006). Different studies have revealed that the most aneuploidy-sensitive are chromosomes 13 (Magli et al., 2006), 15 (Gutierrez-Mateo et al., 2004b), 16 (Pujol et al., 2003), 21 (Cupisti et al., 2003; Kuliev et al., 2005, 2011) and 22 (Clyde et al., 2003; Kuliev et al. 2003, 2011; Sandalinas et al., 2002). These chromosomes, together with chromosomes 1, 7 and 17, are also commonly diagnosed as aneuploid in early embryos (Munne & Cohen, 1998).

Similarly, in IVM oocytes some chromosomes are more frequently affected than others. Aneuploidy rate may increase with prolonged *in vitro* cultivation. This is most notable in chromosomes 18 and 22 which are 6- and 4.4-times more frequently involved in aneuploidies in oocytes with 36-hours maturation period compared to those with 24-hours maturation period, respectively (Table 4D), suggesting that these two chromosomes may be extremely sensitive to cultivation conditions (Križančić Bombek L. et al., 2011).

From the data obtained for aneuploidies of individual chromosomes and on the assumption that aneuploidies approximately equally affect all 23 chromosomes of the human genome, an average and total aneuploidy rate can be estimated (Table 4E). The later is significantly higher in IVM oocytes requiring longer time to achieve maturity (79.6 %) than in those reaching M II phase in less than 24 hours (46.7 %). However, this estimate is most probably somewhat exaggerated since the five tested chromosomes are among those most frequently found in aneuploidies.

IVM oocytes are more prone to different chromosomal aberrations since they are exposed to suboptimal maturation conditions (Chian and Tan, 2002; Chian et al., 2004a; Trounson et al., 2001) and *in vitro* aging (Warburton, 2005). Also IVM in a simple medium may not provide all necessary growth signals and factors which might influence meiotic processes, resulting in aneuploidies and other chromosomal aberrations. During IVM, oocytes undergo maturation phases from GV to MII that can be easily characterized. Usually the oocytes are considered mature, when MII phase is reached, i.e. the PB1 extruded. However, this is only an indicator of nuclear maturity. From the mare presence of PB1, nothing can be deduced about the oocyte's cytoplasmic maturity or chromosome organization. In fact, increased incidence of oocytes with non-organized meiotic-spindle microtubules (43.7) and irregularly arranged chromosomes (33.3 %) can be found among IVM oocytes from stimulated ovarian cycles compared to *in vivo* matured oocytes among which only 13.6 % of cells have non-organized microtubules and 9.1 % have irregularly arranged chromosomes (Li et al., 2006). Our data show similar results (Table 5) with 30.6 % of *in vitro* matured GV oocytes having abnormal shape of meiotic spindle (Figure 8) which is significantly higher than in donated fresh mature oocytes among which only 6.1 % of oocytes show abnormal spindle. Furthermore, in 29 % of IVM oocytes with normal meiotic spindle, chromosomes are irregularly arranged within the spindle itself (Wang & Keefe, 2002).

		24 hours	36 hours
No. of analyzed chromosomes		325	185
A			
Unbalanced premature chromatid separation	Hypohaploidy (%)	8 (2.46)	1 (0.54) [a]
	Hyperhaploidy (%)	6 (1.85)	8 (4.32) [a]
	Total	14 (4.31)	9 (4.86) [i]
Chromosome non-disjunction	Nullisomy (%)	7 (2.15)	8 (4.32)
	Disomy (%)	12 (3.69) [b]	15 (8.11) [b]
	Total	19 (5.84) [j]	23 (12.43) [i, j]
B			
Unbalanced premature chromatid separation (hypohaploidy + hyperhaploidy) (%)		14 (4,31)	9 (4,86) [c]
Chromosome non-disjunction (nullisomy + disomy) (%)		19 (5,85) [d]	23 (12,43) [c,d]
C			
Genetic material loss in oocyte (hypohaploidy + nullisomy)		15 (4.62)	9 (4.86) [f]
Genetic material excess in oocyte (hyperhaploidy + disomy)		18 (5.54) [e]	23 (12.43) [e, f]
D			
Chromosomes with aneuploidy (%)	Chromosome 13	7 (2.15)	6 (3.24)
	Chromosome 16	13 (4.00)	5 (2.70)
	Chromosome 18	2 (0.62) [g]	7 (3.78) [g]
	Chromosome 21	7 (2.15)	4 (2.16)
	Chromosome 22	4 (1.23) [h]	10 (5.41) [h]
E			
Average aneuploidy rate*		2.03	3.46
Total aneuploidy rate		46.7 [k]	79.6 [k]

Table 4. Occurrence of different aneuploidy types with respect to IVM duration. FISH analysis (MultiVysion® PB, Vysis) of chromosomes 13, 16, 18, 21 and 22 in IVM oocytes.
[a-d] $p < 0.05$ (chi-square test with Yates correction)
[e-j] $p < 0.01$ (chi- square test with Yates correction)
[k] $p < 0.05$ (chi- square test)
* based on the five analyzed chromosomes

		Normal spindle	Abnormal spindle
Meiotic stage before and after IVM	M I to M II (n = 31)	25 (80.6%)	6 (19.4%)
	GV to M II (n = 36)	25 (69.4%)	11 (30.6%) [a]
	M I arrest (n = 21)	12 (57.1%)	9 (42.9%) [b]
	Fresh M II (n = 33)	31 (93.9%)	2 (6.1%) [a, b]

Table 5. Configuration of meiotic spindle of *in vitro* matured prophase I (GV) and metaphase I (M I) human oocytes. [a] $p < 0.01$; [b] $p < 0.005$

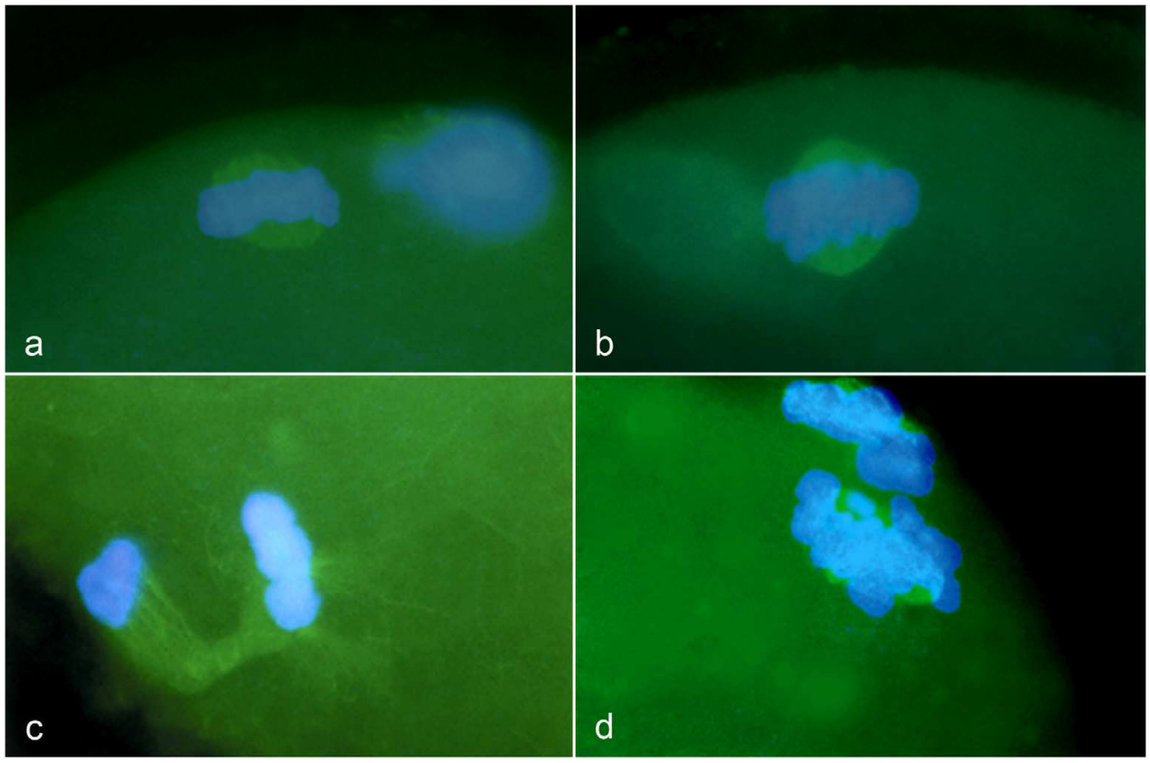


Fig. 8. Second meiotic spindles in metaphase II oocytes after IVM.
Many *in vitro* matured oocytes have normal barrel-shaped second meiotic spindles with chromosomes aligned in equatorial plane (a, b). On the right side of (a), chromosomes of PB1 are visible in a different focal plane. In IVM oocytes with abnormal spindle chromosomes can segregate between the oocyte and PB1 but microtubules remain attached to both chromosome masses instead of forming a proper meiotic spindle in the oocyte (c). It is also possible that meiotic spindles are disrupted or multipolar (d).

6. Conclusion

IVM oocytes can be used in clinical ART cycles however it has to be kept in mind that at least among those oocytes which acquire maturity later the aneuploidy incidence is very high. Therefore, for safer clinical use preimplantation genetic diagnosis and the selection of chromosomally normal embryos is recommended before embryo transfer into the uterus. Such selection significantly increases implantation rate as well as decreases the frequency of spontaneous abortions and birth of affected children.

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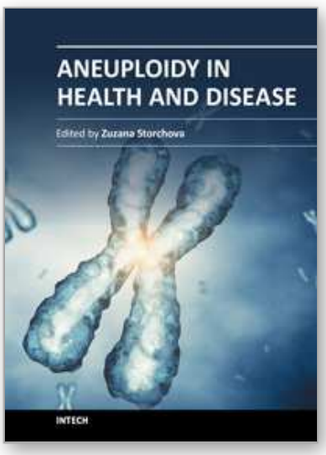
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Aneuploidy means any karyotype that is not euploid, anything that stands outside the norm. Two particular characteristics make the research of aneuploidy challenging. First, it is often hard to distinguish what is a cause and what is a consequence. Secondly, aneuploidy is often associated with a persistent defect in maintenance of genome stability. Thus, working with aneuploid, unstable cells means analyzing an ever changing creature and capturing the features that persist. In the book Aneuploidy in Health and Disease we summarize the recent advances in understanding the causes and consequences of aneuploidy and its link to human pathologies.

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