The Status of the Chromatin of Human Preimplantation Embryos with Good Morphology

(chromatin / cell fragmentation / FISH / IVF / preimplantation embryo)

S. M. DELIMITREVA

Human IVF Laboratory, Department of Biology, Medical Faculty, Sofia, Bulgaria

Abstract. The data about the relation and succession of blastomere fragmentation, cleavage rate and chromatin anomalies in preimplantation mammalian embryos are empirical and controversial at present. In this work we studied the proportion of nuclear fragmentation and condensation in 3–5-cell stage human embryos with no or minimal blastomere fragmentation (morphological class A and B, respectively) and the possibilities to perform FISH chromosomal analyses with them. We observed different stages of chromatin damage in blastomere nuclei corresponding to the steps of nuclear apoptotic changes well known in many cell types. The ploidity analysis of chromosomes 1, 5, 19 and X was determined as successful in embryos which had at least 2 out of 3, 3 out of 4 or 3 out of 5 normal nuclei with an equal number of FISH signals. There was no difference in the percentage of abnormal nuclei among the A- and B-class embryos. Tendencies noted by us suggest that the minimal blastomere fragmentation (up to 20% of perivitelline space) does not preclude the normal nuclear status allowing successful ploidy testing. The presence of condensed chromatin is a critical factor for interphase cytogenetic analysis of single early blastomeres.

A substantial part of mammalian embryos are lost before their implantation into the uterus. This applies to both *in vivo* and *in vitro* fertilization (Buster et al., 1985; Munne et al., 1994; Giorgetti et al., 1995). The preimplantation embryo destruction can be provoked by cell cycle errors leading to genomic anomalies, by the *in vitro* fertilization (IVF) procedure *per se*, cultivation conditions and probably some unknown factors. It is accepted that genetic mechanisms of programmed cell death (PCD) with subsequent apoptosis are involved (Hardy et al., 1993; Hoover et al., 1995; Jurisicova et al., 1996). In this way, the rate of aneuploidy, cell and nuclear fragmentation and blastomere number are the main criteria for assessment of *in vitro* derived human preimplantation embryos prior to intrauterine transfer. The data about relations and succession of these phenomena are empirical and controversial at present – according to some investigations the blastomere fragmentation in cleaving embryos illustrates the apoptotic process (Jurisicova et al., 1996; Warner et al., 1998); other studies support the opposite thesis that apoptosis in early preembryos can be a consequence rather than a cause of fragmentation (Antczak et al., 1999); some authors suggest that cell morphology and chromosomal disorders are related, but on the other hand there is no evidence that genome and chromosomal errors impair preimplantation development (Coonen et al., 1994; Alikani et al., 2000).

In our work we studied the proportion of nuclear condensation and fragmentation in 3–5-cell stage human embryos with no or minimal blastomere fragmentation and the possibilities to perform fluorescent *in situ* hybridization (FISH) chromosomal analyses with them.

Material and Methods

The IVF, intracytoplasmic cell injection (ICSI) and embryo transfer procedures were carried out according to the protocols applied in the Human IVF Unit "Technobioassistance", based at the Department of Biology, Medical Faculty of Sofia (Vatev, 1988; Vatev et al., 1993; Vatev et al., 1998). The investigated embryos were donated for research purposes in the cases when more than the required number of embryos with good morphology and adequate mitotic rate were available on the day of embryo transfer (there is no legislation concerning embryo donation and cryopreservation in Bulgaria). The investigation was approved by the Ethical Committee of the Preclinical University Centre – Medical University of Sofia, and the patients gave informed consent for the supernumerary embryos to be used for scientific research.

We used 42 preembryos at the stage of 3–5 cells with no or minimal blastomere fragmentation. All of them originated from normally fertilized two-pronucleated zygotes and the cell division was registered less than 24 hours before fixation (to exclude "arrested" embryos). The embryo grading system with 4 morphological

Received December 14, 2001. Accepted March 7, 2002.

Corresponding author: Stefka M. Delimitreva, Human IVF laboratory, Department of Biology, 1, St. George Sofiisky Street, 1431 Sofia, Bulgaria.

Abbreviations: FISH – fluorescent *in situ* hybridization, FITC – fluorescein isothiocyanate, ICSI – intracytoplasmic cell injection, IVF – *in vitro* fertilization, PCD – programmed cell death.

classes from A to D, accepted in many IVF centers, was applied (Hoover et al., 1995; Vandervorst et al., 1998). Twenty of the tested embryos were subdivided in class A (with no blastomere fragmentation) and 22 of them in class B (the volume of cell fragments occupied up to 20% of the perivitelline space).

Zonae pellucidae were removed by Tyrode solution, pH 2.5, adjusted by HCl. The embryos were incubated for 20 min at room temperature in hypotonic solution (1% sodium citrate with 5% bovine serum albumin (BSA) to prevent adhesion to the dish), dropped in minimal volume onto slides immediately cleaned by acetone and fixed by methanol/acetic acid (3 : 1). Fixation was controlled under a phase-contrast microscope. The fixative was dropped until the complete disappearance of the cytoplasm. FISH was carried out according to Munne et al. (Munne et al., 1993; Delimitreva et al., 2001). Directly FITC (fluorescein isothiocyanate)-labelled probes (Boehringer Mannheim GmbH, Mannheim, Germany) specific for centromeric regions of chromosomes 1, 5, 19 were denatured simultaneously with nuclear DNA and hybridized for 5 h. The nuclei were counterstained with propidium iodide (1 mg/ml) diluted in glycerol/PBS (9 : 1). Antifade reagent DABCO (3% diazabicyclo[2.2.2]octane – Sigma, St. Louis, MO) was supplied and fluorescence of DNA was observed by Zeiss Aksioscop 20 (Zeiss, Oberkochen, Germany). After washing in PBS, pH 8, with 1% Nonidet-P40 (Sigma) and dehydration in ethanol (70, 85 and 100%), the same hybridization procedure for detection of the X chromosome was performed using FITC-labelled centromeric probes.

Results

A hundred and fifty-six blastomeres were tested. The nuclei of 94 of them with typical interphase chromatin with homogeneous fluorescence or mitotic chromosomes were classified as normal. Successful FISH reaction was registered in all normal nuclei (Fig. 1). Fragmented or flocculent nuclei were observed in 34 blastomeres. In five of them there were 2–3 separate fragments in a blastomere, up to 8 flocculi were joined like clover leaves in the others. When the chromatin appeared relatively homogeneous, some FISH signals were visible in the fragments, but their number was chaotic and was not usable as a result (Fig. 2). Twenty-eight blastomeres had unfragmented nuclei at different stages of condensation. No FISH signals were seen in the nuclei or nuclear fragments at any stage of chromatin condensation.

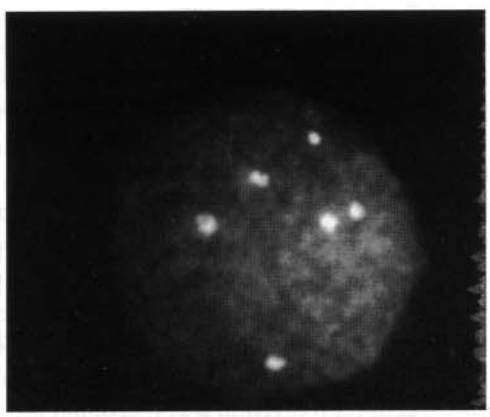

Fig. 1. FISH reaction for chromosomes 1, 5 and 19 (6 spots) in an interphase blastomere nucleus

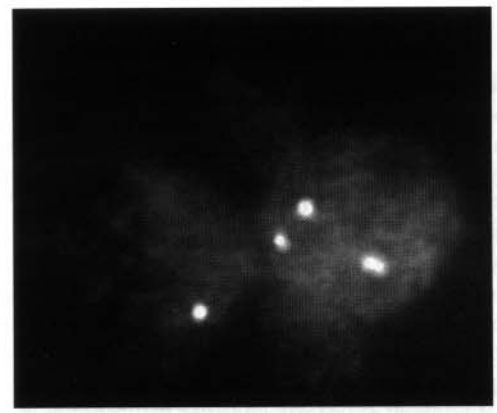

Fig. 2. Abnormal number of FISH signals for chromosomes 1, 5, and 19

According to our observations, the probable chronology of chromatin condensation in the blastomere of an early cleaving human embryo is the following: the fluorescence becomes uneven, the chromatin forms at first a fine network, later the structure becomes rougher, relatively large bright and dark regions appear in the nuclei (Fig. 3a); the contrasting parts increase their size, but links between them are still visible (Fig. 3b), the nuclear volume decreases significantly at this stage; in the next stage the chromatin disperses into small oval dense pieces (Fig. 3c). In fact, these pictures correspond to the steps of nuclear apoptotic changes well known in many cell types (Mohr and Trounson, 1982; Kerr et al., 1987; Jurisicova et al., 1996).

Although our group of embryos was small, some tendencies were noticed. We did not observe a statistically reliable difference in the percentage of abnormal nuclei among the embryo classes A and B ($P_{(\chi 2)} > 0.05$). The portion of fragmented and condensed nuclei for A-class embryos was 25% and 19%, respectively, and for B-class 19% and 17% (Table 1).

The proportion of successfully tested 3-, 4- and 5-cell embryos was described in Table 2. The ploidy analysis of tested chromosomes was determined as successful in 32 embryos, which had at least 2 out of 3, 3 out of 4 or 3 out of 5 normal nuclei with an equal number of FISH signals. Two of them had an abnormal number of some of the identically labelled 1, 5 and 19 chromosomes. Mosaic distribution of the X chromosome was found in four embryos and in one embryo, chromosomes 1, 5 and 19 were distributed chaotically.

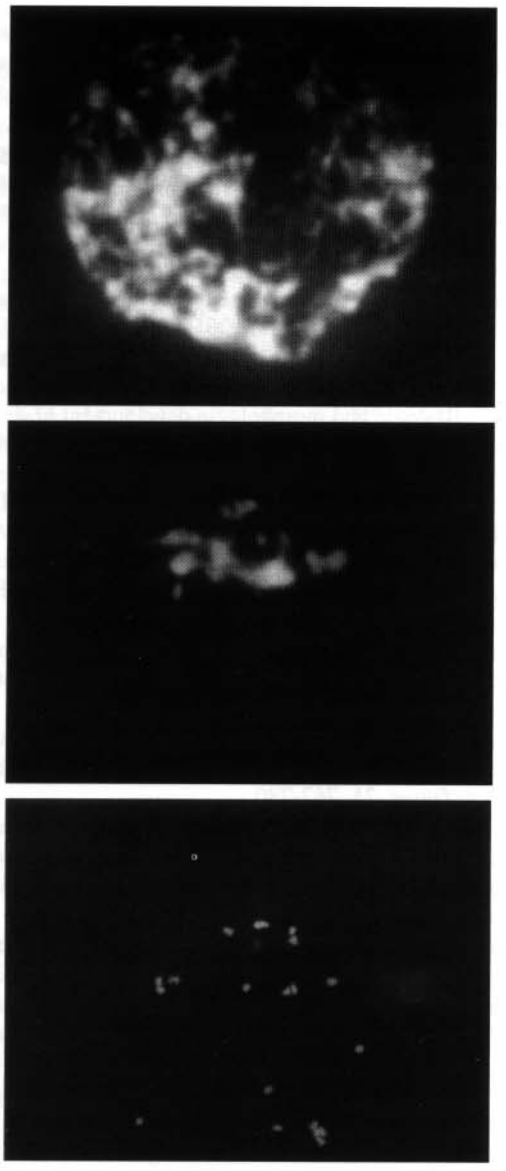

Fig. 3. Subsequent stages of chromatin condensation in blastomere nuclei
A/ an early stage of chromatin condensation;
B/ advanced chromatin condensation;
C/ dense chromatin bodies.

Mosaic and chaotic embryos were excluded from the successfully tested group. The statistical difference in the portion of fragmented and condensed nuclei was reliable only between 4- and 5-cell stages ($0.01 > P_{(\chi 2)} > 0.001$). Nevertheless, the percentage of successfully tested 3-, 4- and 5-cell embryos was similar because of the exclusion of mosaic embryos.

Tendencies noted by us suggest that the minimal blastomere fragmentation (up to 20% of perivitelline space) does not preclude the normal nuclear status allowing successful ploidy testing. The presence of condensed chromatin is a critical factor for interphase cytogenetic investigations in single early blastomeres.

Discussion

Various mitotic rate and morphology patterns can be observed among human preimplantation embryos obtained and cultured at identical conditions and even derived from the same patients using the same protocol for ovarian stimulation. This diversity originates during gametogenesis and cleavage. Probably some disturbances of nucleo-cytoplasmic interactions leading to cell and nuclear abnormalities are involved (Evsikov and Verlinsky, 1998). There are three possible explanations of the link between cell fragmentation and apoptosis in cleaving embryos. First, if apoptotic changes of chromatin at this stage are provoked by blastomere fragmentation, the fragmentation must occur earlier than chromatin condensation; second, if the chromatin damage is the reason for blastomere death, the cell fragmentation must be the later process; finally, these processes might be unrelated. In the literature there are data supporting all of these schemes to be probable in mammalian preembryos (Warner et al., 1998; Antczak et al., 1999). Our results are closer to the first mechanism and do not exclude the last one. The evidence for activity of genes responsible for PCD and early steps of apoptosis in cleaving embryos supports the same idea (Jurisicova et al., 1996). DNA breaking is proved by positive terminal transferase-mediated DNA end labelling (TUNEL) reaction in blastomere nuclei with chromatin appearance similar to these observed by us. Taking into account these data and the pictures of apoptotic nuclei previous-

Table 1. Proportion of abnormal nuclei in class A and B embryos

Class	No. of embryos	No. of tested cells	Fragmented nuclei	Nuclei with condensed chromatin
A 20	75	19 (25%)	14 (19%)	
B 22	81	15 (19%)	14 (17%)	

Table 2. Proportion of successfully tested 3-, 4-, and 5-cell stage embryos

Cleavage stage	No. of embryos	% of fragmented nuclei	% of condensed nuclei	Embryos with determined ploidy
3 cells	14	17%	19%	11 (79%)
4 cells	20	15%	10%	15 (75%)
5 cells	8	23%	30%	6 (75%)

ly described in various tissues as well as in mammalian preimplantation embryos (El-Shershaby and Hinchliffe, 1974; Mohr and Trounson, 1982; Kerr et al., 1987), we can accept that the chromatin anomalies registered in our study were related to apoptosis.

It appears that some checkpoints of the cell cycle are eliminated in mammalian preimplantation embryos, allowing accumulation of faults of chromosome distribution. The checkpoints are introduced at some point during cleavage, leading to normal cell cycle control at the blastocyst stage (Hartwell and Weinert, 1989; Evsikov and Verlinsky, 1998). It is supposed that at the morula-blastocyst transition a negative selection starts against aneuploid cells by PCD in order to prevent their involvement in the embryonic inner cell mass. Some critical level of aneuploidy results in self-destruction of the embryo. The percentage of aneuploid and mosaic embryos is substantial at the cleavage stage – from 20 to more than 50% according to different authors (Plachot et al., 1989; Coonen et al., 1994; Handyside, 1996). It is noted that blastomere morphology corresponds to the aneuploidy at this stage, but no relation between the cleavage rate and correctness of chromosome complement has been demonstrated (excluding arrested embryos) (Pellestor et al., 1994; Magli et al., 2000). Therefore, we consider it premature at this stage to discuss our percentage of aneuploid and mosaic embryos. In our work apoptotic changes were observed in blastomeres without surrounding fragments, i.e. in some of the analysed 3–5-cell embryos the injury appears first in the nucleus and it is not necessarily accompanied with poor cell morphology. Nearly one third of blastomeres tested by us had nuclei damaged to some extent. The fact that the embryonic cell number was 3 to 5 was probably significant for our results. We must have in view that the presence of less than 5 blastomeres at the third day post *in vitro* insemination is considered a cleavage anomaly and is related to a negative prognosis for embryo survival (Betteridge, 1995). The authors who perform preimplantation ploidy investigations report FISH failures caused by biopsy of multinucleated or enucleated blastomeres, but they do not comment on them in the context of apoptotic changes of the chromatin. In order to decrease the risk of biopsy of abnormal blastomere, at the reproductive centres applying preimplantation genetic diagnosis (PGD) it is accepted that more suitable for their aim are the embryos with at least 7 cells at the third day post *in vitro* insemination – such mitotic rate is a criterion for good developmental potential and allows biopsy of two blastomeres per embryo (Munne et al., 1993; Vandervorst et al., 1998; De Vos and Van Steirteghem, 2001). It is possible that at the 7–8-cell stage the chromatin damage is more closely related to cytoplasmic fragmentation, making the affected cells easier to identify.

References

Alikani, M., Calderon, G., Tomkin, G., Garrisi, J., Kokot, M., Cohen, J. (2000) Cleavage anomalies in early human embryos and survival after prolonged culture in vitro. *Hum. Reprod.* **14,** 2634-2643.

Antczak, M., Van Blerkom, J. (1999) Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. *Hum. Reprod.* **14,** 429-447.

Betteridge, K. J. (1995) Phylogeny, ontogeny and embryo transfer. *Theriogenology* **44,** 1061-1098.

Buster, J. E., Busillo, M., Rodi, I. A., Cohen, S. W., Hamilton, M., Simon, J. A., Thorneycroft, I. H., Marshall, J. R. (1985) Biologic and morphologic development of donated human ova recovered by nonsurgical uterine lavage. *Am. J. Obstet. Gynecol.* **153,** 211-217.

Coonen, E., J. C. Harper, F. C. S. Ramaekers, J. D. A. Delhanty, A. H. N. Hopman, J. P. M. Geraedts, A. H. Handyside (1994) Presence of chromosomal mosaicism in abnormal preimplantation embryos detected by FISH. *Hum. Genet.* **94,** 609-615.

Delimitreva, S., Zhivkova, R., Vatev, I. (2001) Supernumerary human preembryos provide potential for preimplantation genetic diagnosis. *Folia Biol. (Praha)* **47,** 88-91.

De Vos, A., Van Steirteghem, A. (2001) Aspects of biopsy procedure prior to preimplantation genetic diagnosis. *Prenat. Diagn.* **21,** 767-780.

El-Shershaby, A. M., Hinchliffe, J. R. (1974) Cell redundancy in the zona-intact preimplantation mouse blastocyst: a light and electron microscope study of dead cells and their fate. *J. Emb. Exp. Morphol.* **31,** 643-654.

Evsikov, S., Verlinsky, Y. (1998) Mosaicism in the inner cell mass of human blastocysts. *Hum. Reprod.* **13,** 3151-3155.

Giorgetti, C., Terrou, P., Auquier, P. (1995) Embryo score to predict implantation after in vitro fertilization: based on 957 single embryo transfers. *Hum. Reprod.* **10,** 1427-2431.

Handyside, A. H. (1996) Mosaicism in the human preimplantation embryo. *Reprod. Nutr. Dev.* **36,** 643-649.

Hardy, K., Winston, R. M. L., Handyside, A. H. (1993) Binucleate blastomeres in preimplantation human embryos in vitro: failure of cytokinesis during early development. *J. Reprod. Fertil.* **98,** 549-558.

Hartwell, L. H., Weinert, T. A. (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science* **246,** 629-634.

Hoover, L., Baker, A., Check, J. H., Lurie, D., O'Shaughenessy, A. (1995) Evaluation of a new embryo-grading system to predict pregnancy rates following in vitro fertilization. *Gynecol. Obstet. Invest.* **40,** 151-157.

Jurisicova, A., Varmuza, S., Casper, R, F. (1996) Programmed cell death and embryo fragmentation. *Mol. Hum. Reprod.* **2,** 93-98.

Kerr, J. F. R., Searle, J., Harmon B. V., Bishop, C. J. (1987) Apoptosis. In: *Perspectives on Mammalian Cell Death,* ed. Potten, C. S., pp. 93-128, Oxford University Press, Oxford.

Magli, M. C., Jones, G. M., Gras, L., Gianarolli, L., Korman, I., Trounson, A. O. (2000) Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocyst in vitro. *Hum. Reprod.* **15,** 1781-1786.

Mohr, L. R., Trounson, A. O. (1982) Comparative ultrastructure of hatched human, mouse and bovine blastocysts. *J. Reprod. Fertil.* **66,** 506-509.

Munne, S., Weier, H.-U.G., Stein, J., Grifo, J., Cohen, J. (1993) A fast and efficient method for simultaneous X and Y in situ hybridization of human blastomeres. *J. Assist. Reprod. Genet.* **10,** 82-90.

Munne, S., Alikani, M., Cohen, J. (1994) Monospermic polyploidy and atypical embryo morphology. *Hum. Reprod.* **9,** 506-510.

Pellestor, F., Girardet, A., Andreo, B., Arnal, F., Humeau, C. (1994) Relationship between morphology and chromosomal constitution in human preimplantation embryo. *Molec. Reprod. Dev.* **39,** 141-146.

Plachot, M., Mandelbaum, J., Junca, A. M., de Grouchy, J., Salat-Baroux, J., Cohen, J. (1989) Cytogenetic analysis and developmental capacity of normal and abnormal embryos after IVF. *Hum. Reprod.* **4,** (suppl.), 99-103.

Vandervorst, M., Liebaers, I., Sermon, K., Staessen, C., De Vos, A., Van de Velde H., Van Assche, E., Joris, H., Van Steirteghem, A., Devroey, P. (1998) Successful preimplantation genetic diagnosis is related to the number of available cumulus-oocyte complexes. *Hum. Reprod.* **13,** 3169-3176.

Vatev, I. (1988) Human in vitro fertilization and embryo transfer program at the Medical Academy, Sofia, Bulgaria. *J. In Vitro Fert. Embryo Transfer* **5,** 48-49.

Vatev, I. Ts., Tarlatzis, B. C., Dukaki, C., Bontis, J., Lagos, S., Mantalenakis, S. (1993) Human assisted fertilization by micromanipulation. *Hum. Reprod.* **8,** 121.

Vatev, I., Karagyozov I., Istatkov, M., Srebreva, M. (1998) Successful application of the fallopian sperm perfusion method and intracytoplasmic sperm injection (ICSI) technique for treatment of human infertility. *Balkan J. Med. Genet.* **3,** 115-119.

Warner, C. M., Cao, W., Exley, G. E., McElhinny, A. S., Alikani, M., Cohen, J., Scott, R. T., Brenner, C. A. (1998) Genetic regulation of egg and embryo survival. *Hum. Reprod.* **13,** 178-196.