Article

# Assessment of DNA fragmentation and aneuploidy on poor quality human embryos

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## Abstract

In human assisted reproduction, low embryo quality due to retarded growth and abnormal cellular morphology results in fewer embryos suitable for transfer. This study aimed to assess the extent of DNA fragmentation and aneuploidy in spare slow growing or arrested human embryos. In 19 assisted reproduction cycles, a total of 57 embryos unsuitable for embryo transfer were used for simultaneous apoptosis and aneuploidy assessment. Among them, 31 (54.3%) showed DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) analysis. Among 26 embryos that were negative for TUNEL, interpretable fluorescence in-situ hybridization (FISH) results were obtained for 25 embryos (96.2%). Sixteen embryos were detected to be chromosomally abnormal (64.0%); three were found to be chaotic, six had complex aneuploidy, six had complete monosomy and one was polyploid. The results show that a high level of DNA fragmentation and aneuploidy are common in embryos with slow growth and/or low quality. More detailed studies are needed to assess the effect of factors such as ovarian stimulation regimens and in-vitro culture conditions. Moreover, application of simultaneous TUNEL and FISH techniques can be informative regarding DNA integrity and aneuploidy.

***Keywords:*** *aneuploidy, DNA fragmentation, embryo quality, slow growth*

## Introduction

In human IVF procedures, it is generally observed that during extended in-vitro culture, only a few fertilized oocytes can actually develop into good quality human embryos or blastocysts, whereas the rest show retarded or arrested development as well as abnormal morphology due to unequal cell division or cellular fragmentation. It has been proposed that during extended culture, good quality embryos with superior developmental potential are eliminated from the presumably 'abnormal' ones showing poor embryo quality and development (Huisman *et al*., 1994).

A number of factors have been shown to affect the survival of the embryos beyond 8-cell or up to the blastocyst stage. They include inappropriate stimulation regimens, oocyte maturation and suboptimal culture conditions (Moor *et al*., 1998), maternal age and paternal factors (Janny and Menezo, 1996; Jones *et al*., 1998; Schoolcraft *et al*., 1999), lack of growth factors (Kaye, 1997), and the presence of chromosomal and/or nuclear abnormalities (Munné *et al*., 1995). Some intrinsic factors may also trigger the elimination of embryos with low developmental potential (Hardy, 1999). A large population of human embryos are mosaics, that is, while some cells are diploid, there exist some blastomeres with chromosomal abnormality (Harper *et al*., 1995; Bielanska *et al*., 2002). Depending on the degree of abnormalities as well as the proportion of cells with normal/abnormal chromosomal content, embryo morphology and developmental competence, as well as quality, can vary. It has recently been reported that aneuploidy is usually associated with embryo arrest (Munné *et al*., 1995).

Since cellular and nuclear fragmentation are common observations during apoptosis in a variety of somatic cells, it has been suggested that the appearance of these fragmentation

patterns in preimplantation embryos could also be associated with apoptosis, leading to loss of certain blastomeres or death of a whole embryo (Jurisicova *et al.*, 1996; Levy *et al.*, 1998).

This study was designed to analyse the degree of DNA fragmentation and the extent of chromosomal abnormalities simultaneously in slow growing (or arrested) poor quality human embryos by applying terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) and fluorescence in-situ hybridization (FISH) techniques consecutively on selected embryos. Arrested or slow growing embryos of poor quality have a higher rate of DNA fragmentation and chromosomal abnormality. TUNEL is very advantageous for detecting nuclear fragmentation, since it allows both localization and the quantification of the degree of nuclei with DNA fragmentation. Additionally, embryos having intact nuclear DNA can be processed for multicolour FISH analysis without further procedures or special treatments.

# Materials and methods

## Patients

This study was approved by the Internal Review Board of Istanbul Memorial Hospital and informed consent was obtained from each couple who participated in this study. Fifty-seven embryos from 19 karyotypically normal couples with male infertility were analysed. Patients were selected based on the criteria that they produced mostly (or all) embryos with poor quality, and hence they donated their spare arrested or slow growing embryos that were rejected for embryo transfer. Of the 19 female partners, two were diagnosed as having polycystic ovaries ($n = 2$), two with tubal factor infertility ($n = 2$), four had diminished ovarian reserve (DOR) and the remaining 11 were classified as normal responders. For male partners, nine had oligoasthenoteratozoospermia (OAT), five were diagnosed as severe OAT and five had azoospermia.

## Ovulation induction and oocyte recovery

The stimulation protocols were as outlined previously (Kahraman *et al.*, 2002). Pituitary down-regulation was performed using a gonadotrophin-releasing hormone analogue (Buserelin Suprefact®; Hoechst AG, Frankfurt, Germany) and follicular development was then stimulated with an injection of FSH (Gonal-F®; Serono, Istanbul, Turkey; Puregon®; Organon, Istanbul, Turkey; Metrodin®; Serono), human menopausal gonadotrophin (HMG) (Humegon®; Organon) and human chorionic gonadotrophin (HCG; Profasi, Serono or Pregnyl, Organon).

## ICSI and embryo culture

Transvaginal ultrasound-guided oocyte retrieval was performed 36 h after the injection of 10,000 IU HCG. Approximately 2 h after oocyte retrieval, the cumulus cells and the corona radiata were removed by brief exposure (10 s) to Gamete-20 containing 40 IU/ml hyaluronidase (type VIII, specific activity 320 IU/mg, H 3757®; Sigma Chemical Co., Steinheim, Germany) and intracytoplasmic sperm injection (ICSI) was applied to MII oocytes. Immediately after ICSI, injected oocytes were put into G1.2 medium (Vitrolife, Gothenburg, Sweden) and cultured in this medium until the morning of day 3. On day 3, embryos were taken into G2.2 medium (Vitrolife) and kept in this medium until the day of embryo transfer. Every 24 h, embryos were transferred into fresh medium. Fertilization was assessed at 16–18 h after injection, and was determined as normal when two clearly distinct pronuclei containing precursor nucleolar bodies (PNB) were present under an inverted microscope. The state of embryo cleavage and quality was assessed after a further 24, 48, 72 and 96 h of in-vitro culture. The embryos were evaluated according to the number of blastomeres present, blastomere size equality and the relative proportion of anucleate fragments by at least two experienced embryologists at ×600 magnification on an inverted microscope.

## Embryo manipulation and fixation

Embryos were termed as arrested when no further development was observed after 24 h of extended culture. For fixation, selected embryos were first transferred into a hypotonic solution droplet and treated with HCl–Tween 20. After total digestion, the remaining cytoplasm was removed with methanol/acetic acid (3/1, v/v) solution and the nuclei were fixed onto the slide. The slides were then allowed to air dry at room temperature for 15 min.

## Assessment of DNA fragmentation

DNA fragmentation was assessed by the in-situ Death Detection Kit (1684795; Roche Diagnostics, Mannheim, Germany) with the following protocol. Labelling solution was prepared by mixing 2.5 µl of solution 1 and 22.5 µl of solution 2 and added on each slide, which was then covered with a large coverslip. Slides were then covered by aluminium foil and put into a humidity chamber for incubation at 37°C for 45 min. After incubation, slides were washed with phosphate-buffered saline (PBS) three times (1 min for each), and left for air drying. Before the evaluation, 8 µl of 4,6-diamidino-2-phenylindole (DAPI) was applied to each slide and the slide was examined under a fluorescence microscope. Corresponding images are shown in **Figure 1**. Embryos having no fragmentation on their blastomeres were processed by FISH (shown in **Figure 1a**,**b**). Embryos having partial (**Figure 1c**) or complete fragmentation (**Figure 1d**) were excluded from FISH analysis.

## FISH analysis

After the initial denaturation for 5 min at 73°C the slides were hybridized with 3 µl of probe mix at 37°C. FISH analyses were performed using DNA probes specific for chromosomes 13, 18, 21, X and Y. Following the hybridization step in a hybridization chamber (Hybrite; Vysis, Downer's Grove, IL, USA) at 37°C for nearly 8 h, post-hybridization washes were performed in 0.4× SSC, 0.3% NP40 for 2 min at 73°C and 1 min in 2× SSC, 0.1% NP40 at room temperature. After drying the slides, 10 µl of DAPI was added onto each, and slides were evaluated under a fluorescence microscope (Olympus BX 50; Olympus, Tokyo, Japan), with recommended filters.

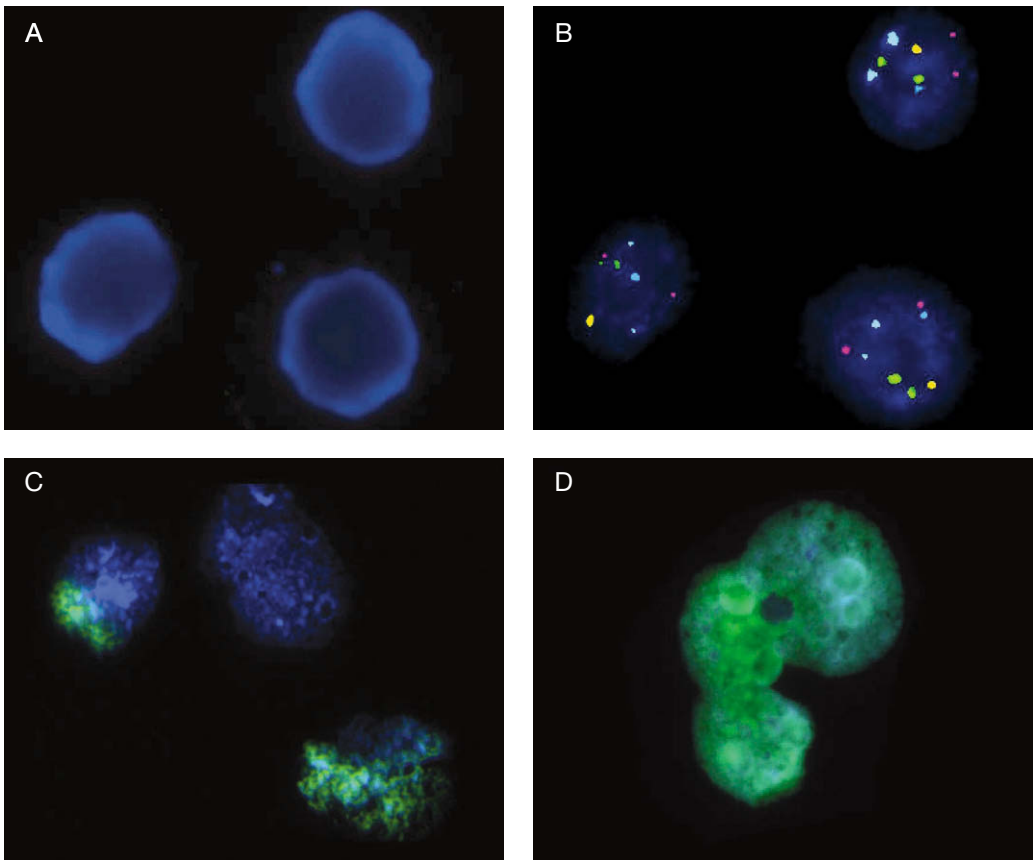


**Figure 1**. (**a**) Blastomeres with intact DNA (no fragmentation), (**b**) corresponding FISH image for three nuclei shown in (**a**), (**c**) partial DNA fragmentation (coloured green), (**d**) complete DNA fragmentation as indicated by green coloured nuclei.

## Results

**Table 1** summarizes the clinical outcome of 19 cycles. Overall, the mean female and male ages were 30.4 ± 5.9 and 37.8 ± 8.0 respectively. The mean duration of infertility was 8.0 ± 4.3 years. Out of 19 cycles, 11.4 ± 5.7 MII oocytes were retrieved on average per cycle, with a fertilization rate of 68.6%. Sixteen embryo transfers were performed, since three cycles were cancelled due to lack of chromosomally normal embryos after PGD ($n$ = 1) and total embryo arrest on day 1 and 3 ($n$ = 2). Five clinical pregnancies were obtained, giving a pregnancy rate of 31.2% (5/16) with an implantation rate of 12.7%.

In this study, 57 poor quality human embryos at different developmental stages from 19 couples undergoing assisted reproduction treatment were individually analysed by simultaneous TUNEL and FISH techniques. As shown in **Table 2**, 31 out of 57 embryos (54.3%) were found to have fragmented nuclear DNA, and were therefore excluded from the consequent FISH analysis. For embryos having an intact nuclear genome, multicolour FISH was successful for 25 embryos (96.2%), of which 16 (64%) were found to have abnormal chromosomal constitution for the chromosomes tested (13, 18, 21, X and Y). Fifteen of these embryos contained aneuploidies (93.7%). Distribution of the observed abnormalities were monosomy ($n$ = 6), complex aneuploidy ($n$ = 6) containing more than two abnormalities on different

**Table 1.** Patient characteristics and clinical outcome.

| *Parameter* | *Number (mean ± SD)* | *Range* |
|---|---|---|
| No. of cycles | 19 | |
| Female age (years) | 30.4 ± 5.9 | 17–41 |
| Male age (years) | 37.8 ± 8.0 | 28–58 |
| Duration of infertility | 8.0 ± 4.3 | 1.5–16 |
| MII oocytes/patient | 11.4 ± 5.7 | 2–24 |
| Fertilization rate (%) | 68.6 | |
| Embryo transfer cycles | 16 | |
| Embryo transfers cancelled | 3 | |
| Lack of normal embryos after PGD | 1 | |
| Low fertilization and developmental arrest | 2 | |
| Day of embryo transfer | | |
| Day 3 | 4 | |
| Day 4 | 10 | |
| Day 5 | 2 | |
| Pregnancies | | |
| Pregnancy rate (%) | 31.2 (5/16) | |
| Implantation rate (%) | 12.7 | |

Values in parentheses are numbers.

chromosomes tested, chaotic constitution ($n$ = 3) and polyploidy ($n$ = 1).

Analysed embryos were additionally classified according to their developmental stages, and the results are shown in **Table 3** and **Table 4**. Among the eight prezygotes found to be arrested on day 1 of embryo development, five contained fragmented DNA (62.5%) and only three could be analysed by FISH. Of the three prezygotes analysed, two were found to have normal chromosomal content and in one both trisomy 18 and monosomy X were detected. From the 20 embryos found to be either arrested on day 2 or slow growing on day 3, DNA fragmentation was observed in 12 (60.0%). For eight embryos to which FISH was applied, only one was found to be normal. All embryos analysed by FISH contained cellular fragmentation ranging from 10 to 20%. As a third group, 21 embryos either arrested on day 3 or slow growing on day 4 were documented, and nine contained fragmented DNA (42.8%). Out of 12 embryos selected for FISH, seven were found to be chromosomally abnormal. Interestingly, almost all abnormal embryos contained a variable degree of cytoplasmic fragmentations, which was rarely observed in embryos with normal chromosomal content. Only eight embryos were analysed for day 4-arrested/day 5-slow growth pattern and of these, five were positive for TUNEL assay (from the same patient). Of three TUNEL-negative embryos, in one FISH was not successful, one embryo was found to be normal, and the other carried monosomy 22.

**Table 2.** TUNEL and FISH results.

| *Embryos* | n | *%* |
|---|---|---|
| Analysed | 57 | |
| With fragmented nuclei | 31 | 54.3 |
| Total fragmentation | 26 | 83.9 |
| Partial fragmentation | 5 | 16.1 |
| Assessed by FISH | 26 | |
| With interpretable FISH data | 25 | 96.2 |
| Total abnormal | 16 | 64.0 |
| Aneuploid | 15 | 93.7 |
| Chaotic | 3 | 18.8 |
| Complex aneuploid | 6 | 37.5 |
| Monosomic | 6 | 37.5 |
| Polyploid | 1 | 6.2 |

The relationship between TUNEL results and degree of cytoplasmic fragmentation of embryos which were found to be TUNEL(+) are documented in **Figure 2**. Ten out of 12 TUNEL(+) embryos, which were either arrested on day 2 or slow growing on day 3, contained cytoplasmic fragmentations ranging from 10 to 50%. For embryos arrested on day 3 or slow growing on day 4, only three out of nine embryos showed cytoplasmic fragmentation of more than 10%. For five TUNEL-positive embryos analysed on day 5, two were 5- and 8-cell and three embryos were at the compaction–morula stage.

The possible role of sperm quality was investigated by subgrouping the analysed embryos according to the indication of the male partner, i.e. as azoospermia, severe OAT and OAT respectively. Results are shown in **Table 5**. In the azoospermia group, 13 embryos were processed for TUNEL assay and FISH. Four were found to contain fragmented DNA and four were chromosomally abnormal. For cases with severe OAT, 12 embryos were analysed, seven were positive for TUNEL and four were chromosomally abnormal. As for the last OAT group, 32 embryos were assayed for DNA fragmentation and chromosomal abnormalities. As a result, 20 embryos were found to contain fragmented DNA and eight were chromosomally abnormal. In order to compare the effect of sperm concentration, the severe OAT and azoospermia groups were also combined and corresponding values are shown in **Table 5**.

The effect of female characteristics and the effect of ovarian stimulation parameters were questioned and documented in detail in **Tables 6**, **7** and **8**. First, the cycles were analysed according to the patient's ovarian reserve and response capacity for a given treatment protocol. In four out of 19 cycles, the female partner had been diagnosed as having DOR, hence a microdose stimulation regimen was applied. In the remaining 15 cases, female partners were mostly normal responders ($n$ = 11) or they had tubal factor infertility ($n$ = 2); two cases were diagnosed as having polycystic ovaries.

DNA fragmentation, aneuploidy and cycle characteristics and assisted reproduction outcome of four poor responder females are documented in **Table 6**. Three of these four patients were young poor responders (female ages were 29, 31 and 34 years old respectively). In two cases, apparent oocyte morphological defects were observed prior to ICSI. In case 1, transparent cytoplasmic structures, termed smooth endoplasmic reticulum

**Table 3.** Distribution of TUNEL and FISH results according to developmental stage. Values in parentheses are percentages.

| *Day of development* | *No. of embryos* | | | |
|---|---|---|---|---|
| | *Analysed* | *TUNEL (+)* | *FISH applied* | *FISH abnormal* |
| Day 1 (prezygote stage) | 8 | 5 (62.5) | 3 | 1 |
| Day 2: arrested, day 3: slow growing | 20 | 12 (60.0) | 8 | 7 (87.5) |
| Day 3: arrested, day 4: slow growing | 21 | 9 (42.8) | 12 | 7 (58.3) |
| Day 4: arrested, day 5: slow growing | 8 | 5 (62.5) | 3 | 1 |

**Table 4.** Distribution of abnormalities according to developmental stages.

| *No.* | *Morphology* | *Fragmentation (%)* | *FISH result* |
|---|---|---|---|
| *Day 1: arrested (prezygote stage)* | | | |
| 1 | _ | _ | Normal |
| 2 | _ | _ | Normal |
| 3 | _ | _ | Trisomy 18, monosomy X |
| *Day 2: arrested or day 3 slow growing* | | | |
| 1 | 4 cells | 10 | Normal |
| 2 | 5 cells | 10 | Chaotic (trisomy 18, monosomy 13, monosomy 21) |
| 3 | 4 cells | 15 | Monosomy 21 |
| 4 | 5 cells | 10 | Monosomy 13 |
| 5 | 4 cells | 10 | Monosomy 18 |
| 6 | 2 cells | 20 | Monosomy X |
| 7 | 4 cells | 15 | Chaotic (tetrasomy, trisomy) |
| 8 | 3 cells | 10 | Chaotic (2n–4n) |
| *Day 3: arrested or day 4 slow growing* | | | |
| 1 | 5 cells | 5 | Normal |
| 2 | 8 cells | 0 | Normal |
| 3 | 6 cells | 5 | Normal |
| 4 | 5 cells | 0 | Normal |
| 5 | 3 cells | 0 | Normal |
| 6 | 5 cells | 15 | Monosomy 18 |
| 7 | 7 cells | 5 | Monosomy 18, monosomy X |
| 8 | 4 cells | 15 | Trisomy 13, trisomy 21 |
| 9 | 7 cells | 10 | Trisomy 13, trisomy 21 |
| 10 | 7 cells | 10 | Trisomy 21, monosomy Y, monosomy 18 |
| 11 | 6 cells | 10 | Trisomy 21, monosomy 13, monosomy 18 |
| 12 | 5 cells | 0 | Tetraploid |
| *Day 4: arrested or day 5 slow growing* | | | |
| 1 | Compaction | | Normal |
| 2 | 10 cells | | Monosomy 22 |
| 3 | Early cavitation | | FISH failure |

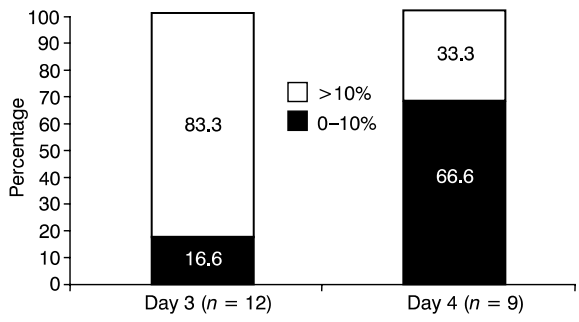


**Figure 2.** Relationship between DNA and cytoplasmic fragmentation as detected by TUNEL assay and embryo morphology evaluation respectively. The upper part of the bars represents embryos with >10% cytoplasmic fragments, the lower part shows the percentage of embryos with <10% fragments.

(SER), were observed for all four fertilized oocytes and all of them failed to cleave during in-vitro culture, hence no transfer was performed for this couple. Similarly, for case 3, all four oocytes contained multiple cytoplasmic vacuoles. Three were fertilized and only one developing embryo could be transferred to the patient, resulting in no pregnancy. For case 2, although oocyte morphology seemed to be normal, the embryos showed a slow cleavage pattern and cytoplasmic fragmentation on day 2. One embryo was found to be arrested on day 3 and the other two contained approximately 40% cellular fragmentation, hence were not selected for embryo transfer. Application of TUNEL assay to these three embryos showed a completely fragmented DNA pattern for the blastomeres analysed. In case 4, the female partner had both diminished ovarian reserve and advanced maternal age. Unfortunately only two oocytes could be retrieved; both were fertilized but one was found to be arrested on day 2. Blastomere biopsy was applied on day 4 for the growing embryo, but the FISH result of the biopsied blastomere was monosomy 22. The embryo transfer was cancelled and the FISH result was confirmed for the whole embryo.

**Table 5.** TUNEL and FISH results according to sperm parameters.

| *Parameters* | *Azoospermia* | *Severe OAT* | *OAT* | *Azoospermia + severe OAT* |
|---|---|---|---|---|
| No. of cycles | 5 | 5 | 9 | 10 |
| No. of embryos analysed | 13 | 12 | 32 | 25 |
| No. of embryos with fragmented nuclei (%) | 4 (30.7) | 7 (58.3) | 20 (62.5) | 11 (44.0) |
| No. of embryos examined by FISH | 9 | 5 | 12 | 14 |
| No. of abnormal embryos using FISH (%) | 4 (44.4) | 4 (80.0) | 8 (66.6) | 8 (57.1) |

OAT = oligoasthenoteratozoospermia.

**Table 6.** Outcomes of assisted reproduction, embyro development, TUNEL and FISH for four poor responder females.

| *Cases studied* | *Oocyte morphology* | *Embryo development on day 2* | *Embryo development on day 3* | *TUNEL* | *FISH* | *Result* |
|---|---|---|---|---|---|---|
| *Case 1* | | | | | | |
| Female: DOR (29 years old), stim. protocol: microdose, male: OAT, MII oocytes collected: 7, fertilized: 4, previous trials: 0 | SER<br>SER<br>SER<br>SER, hgrn | Arrested<br>Arrested<br>Arrested<br>Arrested | | (–)<br>(–)<br>(+)<br>(+) | Normal<br>Complex | No transfer |
| *Case 2* | | | | | | |
| Female: DOR (31 years old), stim. protocol: microdose, male: azoospermia, MII oocytes collected: 4, fertilized:4, previous trials: 1 | Normal<br>Normal<br>Normal<br>Normal | 2 cells, 5%f<br>2 cells, 10%f<br>2 cells, 10%f<br>2 cells, 10%f | 3 cells, 40%f<br>Arrested<br>4 cells, 40%f<br>6 cells, 10%f | (+)<br>(+)<br>(+)<br>NA | (–)<br>(–)<br>(–)<br>Transferred | No pregnancy |
| *Case 3* | | | | | | |
| Female: DOR (34 years old), stim. protocol: microdose, male: OAT, MII oocytes collected: 4, fertilized: 3, previous trials: 2 | Mult.vac<br>Mult.vac<br>Mult.vac | 3 cells, 50%f<br>Arrested<br>4 cells, 10%f | Arrested<br>–<br>5 cells, 10%f | (+)<br>(–)<br>NA | (–)<br>Normal<br>Transferred | No pregnancy |
| *Case 4* | | | | | | |
| Female: DOR (41 years old), stim. protocol: microdose, male: OAT, MII oocytes collected: 2, Fertilized:2, previous trials: 1 | Normal<br>Normal | 3 cells, 5%f<br>Arrested | 5 cells 10%f<br>– | (–)<br>NA | Mon 22[a] | No transfer |

DOR = diminished ovarian reserve; SER = smooth endoplasmic reticulum; OAT = oligoasthenoteratozoospermia; hgrn = homogeneous cytoplasmic granulation; %f = % fragmentation; Mult.vac = multiple cytoplasmic vacuoles; Mon = monosomy; NA = not analysed.
[a]Blastomere biopsy performed on day 4 and the result was confirmed on whole embryo after embryo transfer was cancelled.

Abnormalities observed on oocyte morphology were more evident when normal responder patients were retrospectively analysed. As shown in **Table 7**, in five cycles, specific oocyte morphology defects were detected in the majority of the oocytes retrieved, such as centrally granulated or homogeneously dark cytoplasm, large or narrow perivitelline space and irregular oocyte shape combined with multiple perivitelline debris, each being specific for a given cycle. Furthermore, no pregnancy resulted in four out of five transfers. For case 3, only one out of eight MII-stage injected oocytes was found to be fertilized (12.5%); however, this fertilized zygote was observed to be blocked on day 3 with three blastomeres having fragmented DNA.

As mentioned above, although the majority of embryos in the cycles analysed in this study exhibited poor embryo quality characteristics such as slow cleavage rates, cytoplasmic fragmentation and/or granulation, suitable embryos were available for transfer in 16 out of 19 cycles and embryo transfers resulted in five clinical pregnancies. Cycle characteristics of these couples and the results are documented in **Table 8**. Microdose stimulation protocol was used in all five cycles. Except for case 1, no major oocyte morphology defects were observed. It should also be noted that for case 3, an ongoing pregnancy was obtained after seven unsuccessful assisted reproduction attempts. In all of the previous cycles, starting from day 2, uniform fragmentation was observed in nearly all embryos, resulting in poor quality embryos for embryo transfer. Moreover, preimplantation genetic diagnosis (PGD) was also applied in previous cycles to delineate the factors that might be associated with chromosomal abnormalities. In the last cycle, autologous endometrial co-culture was used and five of the developing embryos on day 4 were transferred to the patient.

**Table 7.** Outcomes of assisted reproduction, TUNEL and FISH for normal responder cases with abnormal oocyte morphology.

| *Cases studied* | *No. of oocytes* | | | *No. of embryos* | | | | *Result* |
|---|---|---|---|---|---|---|---|---|
| | *Retrieved* | *MII* | *Fertilized* | *Analysed* | *TUNEL (+)* | *FISH* | *Transferred* n *(day)* | |
| *Case 1. Centrally granulated cytoplasm* | | | | | | | | |
| Female: 29 years old, stim. protocol: minidose, male: OAT (39 years old), previous trials: 0 | 23 | 16 | 8 | 2 | 1 | 1 (normal) | 5 (day 4) | No pregnancy |
| *Case 2. Large perivitelline space* | | | | | | | | |
| Female: 28 years old, stim. protocol: minidose, male: azoospermia (30 years old), previous trials: 0 | 20 | 15 | 12 | 4 | 3 | 1 (abnormal) | 4 (day 3) | No pregnancy |
| *Case 3. Irregular oocyte shape + perivitelline debris* | | | | | | | | |
| Female: 26 years old, stim. protocol: minidose, male: OAT (32 years old), previous trials: 0 | 13 | 8 | 1 | 1 | 1 | 0 | - | No transfer |
| *Case 4. Narrow perivitelline space* | | | | | | | | |
| Female: 28 years old, stim. protocol: minidose, male: OAT (30 years old), previous trials: 0 | 21 | 12 | 8 | 4 | 4 | 0 | 4 (day 4) | No pregnancy |
| *Case 5. Dark cytoplasm* | | | | | | | | |
| Female: 26 years old (tubal factor), stim. protocol: minidose, male: OAT (32 years old), previous trials: 4 | 23 | 14 | 10 | 5 | 0 | 5 (1 normal, 4 abnormal) | 2 (day 4) | No pregnancy |

**Table 8.** Outcomes of assisted reproduction, TUNEL and FISH applications for pregnant couples.

| *Cases studied* | *No. of oocytes* | | | *No. of embryos* | | | | *Result* |
|---|---|---|---|---|---|---|---|---|
| | *Retrieved* | *MII* | *Fertilized* | *Analysed* | *TUNEL (+)* | *FISH* | *Transferred* n *(day)* | |
| *Case 1. Oocyte morphology: narrow perivitelline space* | | | | | | | | |
| Female: 18 years old, stim. protocol: minidose, male: severe OAT (45 years old), previous trials: 0 | 27 | 12 | 7 | 3 | 2 | 1 (abnormal) | 3 (day 3) | Pregnant |
| *Case 2. Oocyte morphology: normal* | | | | | | | | |
| Female: 28 years old, stim. protocol: minidose, male: azoospermia (32 years old), previous trials: 0 | 16 | 16 | 11 | 3 | 0 | 3 (2 normal, 1 FISH failure) | 4 (day 5) | Pregnant |
| *Case 3. Oocyte morphology: normal* | | | | | | | | |
| Female: 30 years old, stim. protocol: minidose, male: OAT (43 years old), previous trials: 6 | 30 | 24 | 17 | 8 | 6 | 2 (2 abnormal) | 5 (day 4) | Pregnant |
| *Case 4. Oocyte morphology: normal* | | | | | | | | |
| Female: 30 years old, stim. protocol: minidose, male: azoospermia (34 years old), previous trials: 0 | 19 | 15 | 15 | 1 | 0 | 1 (normal) | 5 (day 4) | Pregnant |
| *Case 5. Oocyte morphology: normal* | | | | | | | | |
| Female: 31 years old (tubal factor), stim. protocol: minidose, male: Severe OAT (38 years old), previous trials: 1 | 10 | 8 | 8 | 4 | 4 | 0 | 3 (day 4) | Pregnant |

When the remaining five cycles were retrospectively analysed, no distinct morphological abnormality was observed in the oocytes. Other than having abnormal sperm parameters and two of the female partners having polycystic ovaries, there was no clear indication related to partners' age, hormonal parameters, or number of oocytes obtained or fertilized. However, for one normal responder, three out of the four embryos analysed showed chromosomal abnormalities. These abnormalities were monosomy 18, monosomy 13 and combined monosomies of chromosomes 18 and X respectively.

## Discussion

Numerous embryo grading systems have been defined to select embryos with superior implantation potential in order to increase the overall efficiency of assisted reproductive techniques. Although some have proven their usefulness clinically, they are in fact based solely on morphology observed under the microscope, and do not give any information about the nuclear status of the embryos in question. These systems are also based on the assumption that prolonged culture allows development of normal embryos with implantation potential, while abnormal and non-viable embryos arrest before or shortly after the onset of genomic activation (Huisman *et al*., 1994; Janny and Menezo, 1996; Gardner and Lane, 1997). In parallel with these suggestions, the majority of arrested embryos are in fact found to be chromosomally abnormal (Munné *et al*., 1995). The current results support these previous assumptions and observations to the extent that the majority of the slow growing or arrested embryos were found to contain a high percentage of DNA fragmentation and chromosomal abnormalities. Furthermore, no aneuploidies containing a single trisomy of the chromosomes analysed were observed. In fact, the majority of abnormalities detected were monosomies, or involved multiple

abnormalities containing monosomies and double trisomies. In combination with the results of Bielanska *et al.* (2002), the data suggest that these two forms of chromosomal abnormalities are very common among arrested embryos, and are eliminated during the first and the third cleavages. It is also consistent with the rarity of multiple chromosome abnormalities, and the very low rates of double trisomies found among spontaneous abortions (Reddy, 1997).

Several studies performed on embryos in PGD cycles show that some embryos with abnormal chromosomal structure (mostly trisomies and monosomy X) can successfully reach the blastocyst stage and implant to the uterus, whereas some chromosomally normal embryos, although they have good morphology, may fail to undergo differentiation in extended culture (Magli *et al.*, 2000; Sandalinas *et al.*, 2001; Causio *et al.*, 2002). In this study, some arrested embryos had intact DNA structure and normal chromosome content for the five chromosomes analysed. A common observation in these embryos was that they did not show significant cellular fragmentation, and all arrested between the 3- to 8-cell stages. However, whether they contained abnormalities on other chromosomes was not determined.

It has been suggested that most of the chromosomal abnormalities can arise during oogenesis, and cell cycle checkpoints are not operational at this stage or during early cleavage divisions (Handyside and Delhany, 1997; LeMarie-Adkins *et al.*, 1997; Harrison *et al.*, 2000; Kuliev *et al.*, 2003). In that sense, these errors could be related to patient-specific factors such as advanced maternal age, or as a result of suboptimal culture conditions, or due to the effect of change in temperature and reactive oxygen species (Pickering *et al.*, 1990; A'arabi *et al.*, 1997; Yang *et al.*, 1998; Gianaroli *et al.*, 2001; Munné *et al.*, 2002, 2003).

Compromised preimplantation development in embryos having abnormal cleavage properties such as cytoplasmic fragmentation and multinuclation has been shown in several studies (Gioretto *et al.*, 1995; Alikani *et al.*, 2000). Current literature also shows that DNA fragmentation to some degree exists in gamete cells, throughout the preimplantation stage and especially together with embryos having cytoplasmic fragmentation. Electron microscopy studies confirm the typical morphological features of apoptosis, implying that programmed cell death may be triggered before the blastocyst stage (Jurisicova *et al.*, 1996; Lopes *et al.*, 1998; Antczak and Van Blerkom, 1999; Hardy *et al.*, 2001). Overall, the onset of apoptosis coincides with compaction, which is the first differentiative event during embryogenesis.

Sperm aneuploidy and DNA fragmentation have also been studied recently by several groups using fresh or frozen–thawed sperm samples. Compared with controls, these rates were found to increase in patients with impaired semen parameters. Significantly higher DNA fragmentation was also observed in patients with testicular cancer and after the application of certain freezing protocols. Although there was no correlation with the fertilization rate, a strong correlation has been found among increased sperm aneuploidy, DNA fragmentation and poor pregnancy outcome (Gandini *et al.*, 2000; Aitken *et al.*, 2003; Calogero *et al.*, 2003; Henkel *et al.*, 2003; Thompson-Cree *et al.*, 2003). Since all of the male partners in this study were diagnosed as having male infertility ranging from OAT to azoospermia, the possible negative effect(s) of relatively poor sperm parameters and morphology on poor embryo quality cannot be excluded. Unfortunately, neither aneuploidy nor DNA fragmentation was studied in this male population, except for one case. However, it was possible indirectly to compare the TUNEL and FISH percentage in two groups: one was the OAT group and the second group was the combination of two data sets including azoospermia and severe OAT. Similar rates for both TUNEL and FISH results were obtained in these groups. However, a more detailed study is needed to assess the contribution of spermatozoa in these cases. Recently, FISH and TUNEL tests have been performed on spermatozoa for selected groups of patients such as patients with severe teratozoospermia, repeated implantation failure and recurrent abortion. Higher rates of sperm aneuploidy observed in these cases may imply that genetic counselling should be given and PGD should be offered in these cases (data not shown).

Although the presence of increased DNA fragmentation in this study and the absence in others is intriguing, the very high incidence of chromosomal abnormalities observed in the same group of embryos can help to explain such a difference. According to the present results, most of the abnormalities detected were monosomies and complex aneuploidies. It is well known that the majority of monosomic embryos cannot go beyond the 8-cell stage and, as has recently been found in mice, that certain chromosomal abnormalities such as haploidy induce an increased incidence of DNA fragmentation and apoptosis (Liu *et al.*, 2002). It is possible that the mechanism causing developmental blockage can trigger a downstream cascade of events that eliminates the embryo by triggering suicide mechanisms. Alternatively, this blockage can also result in abnormal cellular metabolism, leading to embryo death by necrosis. As Hardy stated, the persistence of cytoplasmic fragments that are not cleared by phagocytosis and the increasing amount of DNA degradation that occurs with prolonged arrest indicate that the ultimate fate of arrested embryos can be secondary necrosis (Hardy, 1999). During prolonged culture, similar morphological and nuclear changes have been observed in most arrested embryos. Therefore, it can be argued that the high levels of DNA fragmentation observed in the present study are a consequence, rather than a cause of cell cycle arrest.

Inappropriate stimulation protocols and the quality of the gamete cells are among the other factors that can affect embryo development. In this study, the patient's ovarian reserve, selected stimulation protocol(s), morphology and the maturity of the oocytes produced might be the key parameters for the resulting poor embryo quality. For the four cycles in DOR patients who underwent microdose stimulation, forcing the ovaries with high dose of gonadotrophins might stimulate chromosomally abnormal or developmentally incompetent oocytes to enter the cohort. Although a similar stimulation protocol was followed, various other oocyte morphology defects were also observed in the majority of the oocytes for normal responder females. Some of these abnormalities, such as narrow or large perivitelline space, are thought to be directly related to oocyte maturity and the length of gonodotropin administration. As another type of oocyte abnormality, previous studies have reported the impaired development

profile and the increased chromosomal abnormality rate for oocytes with 'centrally granulated cytoplasm' as well as for oocytes having a dark cytoplasmic appearance (Kahraman *et al*., 2000). Hassan Ali *et al*. recently reported that perivitelline space granularity or debris can be related to oocyte maturity, and this phenomenon can be enhanced with increased dose of HMG used in stimulation. (Hassan Ali *et al*., 1998). Therefore, when combined with the results of DOR patients, the major cause of poor embryo development in this study seems largely related to the quality and the quantity of the oocytes produced.

In the light of this previous research and the present results, it is proposed that high rates of DNA fragmentation and chromosomal abnormalities can, to some extent, be linked to oocyte morphological dysmorphisms. Arrested embryos showing DNA fragmentation at early cleavage stages can inherit this pattern from one or both of the gamete cells. However, in arrested embryos with DNA fragmentation in later stages until the onset of apoptosis like compaction, this pattern is probably generally the result of prolonged culture and/or necrotic events related to high order chromosome abnormalities. As yet, the exact role of apoptosis in preimplantation development is unknown and the nature of these events during preimplantation development needs to be determined. It can be further argued that intrinsic patient properties as well as stimulation protocols themselves can lead to compromised embryo development, DNA fragmentation and increased chromosome abnormalities. Patients having polycyclic ovaries may produce eggs of low developmental potential, possibly due to increased testosterone in the follicular environment. Likewise, vigorous stimulation protocols applied for females with DOR usually result in oocytes with distinct cytoplasmic as well as nuclear abnormalities. Although there were only four cases in this study, the results of DOR patients may be very helpful when alternative stimulation strategies are considered or when counselling for possible assisted reproduction treatment is given to patients having this type of infertility profile.

In conclusion, a great deal of attention has been focused on embryo culture media and conditions and it is usually the culture systems that are questioned first when abnormal embryo development is observed. However, the present results show that most developmentally blocked or poor quality embryos have intrinsic problems that can probably be traced back to gamete cell environment. Furthermore, in some clinics, there is a tendency to perform embryo transfer for poor responder patients even if limited embryos with abnormal cleavage pattern are observed. It is suggested that in such cases, the patient should be informed about the relative status of these embryos, possibility of chromosomal abnormalities and pregnancy outcome. Hence, transfer of arrested or developmentally blocked embryos should be avoided. It is also possible that these embryos can carry some other unknown genomic defects regarding patient characteristics (Beever *et al*., 2003). Together with the chromosomal studies, more research needs to be performed regarding the molecular genetics of these embryos.

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