

BRIEF COMMUNICATION

Impact of blastomere biopsy and cryopreservation techniques on human embryo viability

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The purpose of the present study was to evaluate the effect of cryopreservation on 55 embryos which had one blastomere biopsied for preimplantation genetic diagnosis of aneuploidy before freezing. The thawing outcome was compared to that obtained in 94 embryos which derived from our conventional freezing programme in patients with comparable characteristics who were treated in the same period. Their embryos were morphologically similar but the incidence of aneuploidy was 100% in the biopsy group and unknown in the controls. The percentage of embryos which survived intact after thawing was significantly lower in the biopsied group compared to the controls (9 versus 25% respectively; $P < 0.025$), whereas the rate of lysis was superior among biopsied embryos (34 versus 13% in the controls; $P < 0.001$). Similarly, the survival index was higher in the frozen-intact embryos than in the embryos which were frozen after biopsy (61 versus 38%; $P < 0.001$). No empty zonae resulted in the control group, while six were found after thawing biopsied embryos. In the second part of the study, blastomere biopsy was implemented on 102 thawed embryos generated by 16 patients. The chromosomal analyses revealed that 49 were normal, leading to the transfer of 2.5 ± 0.8 embryos per patient. Only three clinical pregnancies were obtained, and are presently ongoing. In conclusion, the present findings discourage the use of conventional cryopreservation protocols in strategies involving preimplantation genetic diagnosis in human reproductive medicine. Adequate protocols are required for freezing and thawing embryos which have been subjected to biopsy procedures.

Key words: aneuploidy/cryopreservation/embryo implantation/FISH/preimplantation genetic diagnosis

Introduction

Preimplantation genetic diagnosis (PGD) in combination with in-vitro fertilization (IVF) techniques allows for the screening of oocytes and embryos in couples at risk of conceiving

affected pregnancies. Healthy normal babies have been born after PGD of linked diseases (Handyside *et al.*, 1990; Grifo *et al.*, 1992), single gene defects (Handyside *et al.*, 1992), aneuploidy (Munné *et al.*, 1995; Verlinsky *et al.*, 1996; Magli *et al.*, 1998a) and recently after PGD of translocations (Munné *et al.*, 1998). The most common method of PGD entails the biopsy of one or two cells from day 3 embryos; the procedure does not adversely affect embryo development, since at that stage all cells are undifferentiated and still totipotent.

PGD of aneuploidy is routinely performed in our centre by using the multicolour fluorescence in-situ hybridization (FISH) technique on patients presenting with a poor prognosis of pregnancy. This term designates a heterogeneous category of couples whose probabilities of delivering after IVF are consistently low. A possible cause of this reproductive failure may be that the embryos have an increased incidence of numerical chromosomal abnormalities despite having normal morphology and regular developmental rate (60% of the embryos in poor prognosis patients are aneuploid; Gianaroli *et al.*, 1997a). After FISH diagnosis, the feasibility of identifying and transferring euploid embryos has an immediate impact on embryo implantation, which is higher after chromosomal analysis compared to the controls (Gianaroli *et al.*, 1997b). In addition, the number of embryos being cryopreserved is dramatically reduced due to the low number of chromosomally normal embryos which are generally available in these patients' cohorts. However, in some cases supernumerary embryos are present and there is the need to resort to cryopreservation, especially since the high embryo viability associated with euploid embryos requires fewer embryos being transferred per patient. Animal studies have revealed that biopsied and cryopreserved mouse 8-cell stage embryos have the same survival and implantation potential than non-biopsied embryos (Snabes *et al.*, 1993).

The aim of this study was to evaluate the effect of the freezing-thawing procedure on biopsied human embryos, in order to estimate the feasibility of their cryopreservation. The analysis was performed on aneuploid embryos which were not considered for transfer; the thawing outcome was compared with the results derived from our conventional freezing programme.

In the second part of the study, results from the implementation of PGD on thawed embryos are presented, in order to assess whether the cryopreservation process combined with blastomere biopsy could affect embryo viability.

Materials and methods

Biopsied embryos – first part of the study

Embryos were obtained from 30 consenting patients (mean age 36.3 ± 3.7 years) out of 99 undergoing IVF treatment cycles combined with PGD of aneuploidy at the SISMER center. Indications to PGD analysis were: (i) maternal age ≥ 36 years; (ii) ≥ 3 previous IVF failures; and (iii) altered karyotype detected in peripheral blood (gonosomal mosaicism, balanced translocations). On day 3 at 62–64 h after insemination, embryos were scored for number and morphology of blastomeres, nuclei appearance and percentage of fragmentation; those presenting regular morphology and cleavage rate were selected for blastomere biopsy. Following FISH analysis, embryos with an abnormal chromosomal complement were considered not transferable. At approximately 70–72 h post insemination, at the time of embryo transfer, their morphology was re-evaluated and those exhibiting ≥ 7 regular blastomeres and $\leq 5\%$ fragmentation were included in the study.

Blastomere biopsy and fixation

Blastomere biopsy was carried out in HEPES-buffered medium overlaid with pre-equilibrated mineral oil. Zona pellucida was chemically breached (acidic Tyrode's solution at pH 2.35) and the selected blastomere gently removed as already described (Munné *et al.*, 1993). If fragments were present in the perivitelline space, they were also removed during the procedure (Magli *et al.*, 1998b). The nucleus was fixed on a glass slide (methanol–acetic acid 3:1), dehydrated in rising ethanol dilutions (70%, 85% and 100%) and incubated with the hybridization solution at 37°C in a humidified chamber, for 4 h (Munné *et al.*, 1996).

Fluorescence in-situ hybridization technique

The multicolour FISH analysis was performed as described, with the only modification due to the inclusion of a chromosome 16-specific probe (Spectrum Green, Vysis Downers Grove, IL, USA) in the hybridization mixture (Munné *et al.*, 1997). Fluorescent probes were detected using an Olympus BX40[®] microscope equipped with a triple band pass filter; the X chromosome signal appeared as blue, the Y as white, the 13 as orange, the 16 as green, the 18 as pink and the 21 as red.

Embryo cryopreservation

At 70–72 h post insemination, chromosomally abnormal embryos with ≥ 7 regular blastomeres and $\leq 5\%$ fragmentation were cryopreserved following the standard propanediol (PROH) technique (Lassalle *et al.*, 1985). Embryos which presented $\geq 20\%$ fragments before blastomere biopsy and fragment removal were excluded from the study. The control group was represented by 94 embryos derived from 43 patients (mean age 36.1 ± 2.8) which underwent conventional treatment during the same period. These embryos were frozen at 70–72 h after insemination; the selection criteria for freezing were: ≥ 8 regular cells with $\leq 20\%$ fragmentation.

Embryo biopsy on thawed embryos – second part of the study

Sixteen patients with PGD of aneuploidy indications (≥ 3 repeated cycles, $n = 9$; altered karyotype, $n = 5$; X-linked disease, $n = 2$) had all their zygotes frozen in the fresh cycle, as they were at risk of ovarian hyperstimulation syndrome (OHSS) (Ferraretti *et al.*, 1997). Blastomere biopsy was performed at 62–64 h post insemination on embryos at the 7- to 8-cell stage developed after thawing and culturing the corresponding zygotes. Blastomere biopsy, nuclear fixation and FISH analysis were carried out as already described.

Table I. Outcome of frozen–thawed biopsied and non-biopsied embryos

	Biopsied embryos	Non-biopsied embryos
Number of patients	30	43
Age (years; mean \pm SD)	36.3 ± 3.7	36.1 ± 2.8
Number of thawed embryos	55	94
intact (%)	5 ^a (9)	24 ^a (25)
lysed (%)	19 ^b (34)	12 ^b (13)
Survival index %	38 ^c	61 ^c

^a $\chi^2 = 4.98$; $P < 0.025$.

^b $\chi^2 = 8.71$; $P < 0.001$.

^c $\chi^2 = 48.11$; $P < 0.001$.

Table II. Fluorescence in-situ hybridization (FISH) analysis and clinical results following blastomere biopsy on thawed embryos

Number of cycles	16
Age (years; mean \pm SD)	34.2 ± 2.9
Number of embryos thawed	114
Number of embryos analysed by FISH	102
normal (%)	49 (48)
abnormal (%)	53 (52)
Number of embryos transferred (mean \pm SD)	40 (2.5 ± 0.8)
Number of transfer cycles	16
Number of clinical pregnancies (%)	3 (19)
Implantation rate ^a (%)	10

^aNumber of gestational sacs with fetal heart beat divided by the total number of embryos transferred.

Statistical analysis

Results were evaluated by χ^2 analysis 2 \times 2 contingency tables, applying the Yates' correction.

Results

In the first part of the study, a total of 181 embryos were analysed by FISH resulting in 112 being identified as chromosomally abnormal (62%). Of them, 55 were selected for cryopreservation on the basis of their morphological appearance. After thawing, only five embryos were intact (9%), whereas 19 (34%) were either lysed ($n = 13$) or had empty zonae ($n = 6$). In the remaining 31 (56%), the survival index, which represents the number of vital blastomeres divided by the total number of blastomeres frozen, corresponded to 38%. This index gave an estimate of the partial damage which occurred as a result of cryopreservation.

Table I compares the results obtained following the thawing of biopsied embryos with those derived from our conventional cryopreservation programme on embryos at the ≥ 8 -cell stage. The two groups analysed were homogeneous in terms of mean maternal age, morphology of frozen embryos, and protocol and timing at which cryopreservation was carried out. In the control group, 24 embryos out of 94 survived intact (25%) and 12 (13%) were lysed. No empty zonae were observed. The survival index in the remaining 58 embryos was 61%.

For the second part of the study, the outcome of embryo biopsy and FISH analysis performed on thawed embryos is shown in Table II. In all, 114 embryos were obtained, of which 102 were selected for FISH diagnosis. The chromosomal analysis revealed that 49 embryos (48%) were normal, and 53

were aneuploid. All patients had embryos transferred (average number 2.5 ± 0.8 euploid embryos), resulting in three clinical pregnancies and an implantation rate of 10%.

Discussion

Numerical chromosomal abnormalities occurring at meiosis, syngamy or during the first cleavage stages of human development are frequent, and result in embryos with reduced implantation potential (Munné *et al.*, 1995). PGD of aneuploidy allows for the identification of these abnormalities in in-vitro generated embryos; the technique is especially advantageous in those patients with a poor prognosis of pregnancy due to their embryos having a high incidence of chromosomal abnormalities (Munné *et al.*, 1995; Gianaroli *et al.*, 1997a, c). As a result, in this group of patients there is generally no need to cryopreserve supernumerary, chromosomally normal embryos.

According to the present study, the standard PROH protocol for embryo freezing is not satisfactory for biopsied embryos. In fact, the comparison with the data obtained from our conventional cryopreservation programme reveals a significantly lower percentage of intact embryos and higher incidence of lysis (Table I). Although the two groups were homogeneous in terms of maternal age, morphology of the cryopreserved embryos and freezing protocol used, aneuploidy was more frequent in the biopsied embryos (100%) than in the controls (unknown). It can be postulated that aneuploidy may have had an effect on the results presented here; however it is important to take into consideration that, according to our experience, the incidence of chromosomally abnormal embryos in these patient categories is notably high (>60%; Gianaroli *et al.*, 1997a). In addition, events of empty zonae occur more frequently compared with the controls. These effects are probably related to the opening in the zona pellucida which can cause an altered diffusion of the cryoprotectant solution compared to intact embryos. The zona pellucida has an important role in embryo viability and variation in its thickness corresponds to an increased chance of implantation, especially in the case of thawed embryos (Cohen *et al.*, 1988). This condition may be an indicator of increased membrane transport and production of enzymes capable of digesting the zona itself in a localized area. The following hatching process represents one of the first signals of polarity in the recently compacted embryo, which leads to the formation of two primary cell lines: the inner cell mass and the trophectoderm.

According to the current data, alteration of the zona integrity, possibly associated with the removal of one blastomere before freezing, is detrimental to embryo viability during cryopreservation. Physical factors involved in the irreversible damage of embryos during the freezing and thawing procedures have been reported (Ashwood-Smith *et al.*, 1988). Consequently, it may be inferred that the glycoprotein coat surrounding the oocyte has a fundamental role in maintaining cellular integrity and shape throughout the steps of dehydration, shrinking and re-hydration involved in freezing and thawing.

Surprisingly, our data suggest that the cryopreservation process also has an effect on embryo viability when blastomere biopsy is performed on thawed embryos, resulting in only

three clinical pregnancies out of 16 cycles transferred (Table II). In fact, the pregnancy rate which is obtained in our centre in patients undergoing PGD on fresh embryos is 28% in the case of repeated IVF failures and 41% in couples with an altered karyotype, whereas in cases at risk of OHSS, the conventional transfer of thawed embryos yields 35% clinical pregnancies. Thus, even in this case, the combination of blastomere biopsy and cryopreservation procedures gives rise to results which are not expected. This effect could be associated with an interference with intercellular junction formation, due to the freezing and thawing process, as suggested by the facility of removing the blastomere in comparison to fresh embryos at the same stage. In addition, the ensuing cell biopsy could contribute a mechanical disarrangement in their subsequent organization. In all cases, if damage occurs to the cytoskeleton because of abnormal exposure to PROH and sucrose, and/or the blastomere biopsy procedure itself, cell shape is not maintained and this causes an alteration in the bilayer associated glycoproteins and transport systems, which results in cellular death.

In conclusion, the combination of blastomere biopsy and cryopreservation adversely affects embryo viability, irrespective of the sequence in which the two procedures are carried out. This is in contrast to what has previously been reported in the mouse, where 8-cell embryos can successfully undergo freezing and biopsy with a survival rate comparable to that obtained in the controls (Wilton *et al.*, 1989; Snabes *et al.*, 1993). In view of these findings, PGD of aneuploidy is recommended to be currently performed on fresh embryos.

Furthermore, the low survival rate in biopsied embryos after freezing and thawing suggests that the implementation of cryopreservation after PGD of aneuploidy should be restricted until adequate protocols for freezing, including ultrarapid methods, are designed. Alternatively, less aggressive methods to open the zona pellucida should be considered in the hope of alleviating chemical stress to the embryo. At the present time, one of the strategies adopted in our centre is to postpone the transfer of biopsied embryos to day 4. This has two advantages: (i) in cases of supernumerary euploid embryos it allows for a better selection based on morphological evaluation, and (ii) it gives more time for the screening of additional chromosomes after overnight re-hybridization with specific probes. This expedient not only keeps to a minimum the need of resorting to embryo cryopreservation, but also enables the screening of a wider panel of chromosomes, thus offering patients with a poor prognosis increased chances of receiving embryos with the highest implantation potential.

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