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Elevated incidence of chromosomally chaotic embryos among frozen-thawed preimplantation embryos

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Abstract

Objective: The aim of the study was to evaluate the effect of cryopreservation on the formation of chromosomal abnormalities in human preimplantation embryos. *Study design*: The chromosomal constitutions of cleavage stage embryos ($n = 61$) were assessed using fluorescent in situ hybridisation (FISH) technique, applying probes for chromosomes 13, 16, 18, 21, X and Y. Study group embryos frozen at zygote or two-cell stage ($n = 29$) were cultured in vitro post-thawing until they reached four- to six-cell stage, after which their chromosomal constitutions were assessed. Control group embryos frozen at four- to six-cell stage ($n = 32$) were analysed immediately after thawing in order to exclude any post-thaw effect. The proportions of genetically normal and abnormal embryos were compared between study and control group. *Results*: The proportions of normal, aneuploid and mosaic embryos were similar in both groups. However, significantly ($P < 0.05$) higher proportion of chaotic embryos in study (24.1%) compared to control group (6.3%) was observed. *Conclusion*: The elevated level of chromosomally chaotic embryos among embryos that had undergone cellular division after thawing as compared to embryos analysed immediately after thawing indicates a potential negative impact of cryopreservation on the formation of chromosomal abnormalities in preimplantation embryos.
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1. Introduction

Human embryo cryopreservation is an essential part of assisted reproduction programs allowing to store supernumerary embryos for use at a later date. The most prominent advantage of embryo cryopreservation is that it provides means by which the number of replaced embryos can be reduced in both fresh and frozen embryo transfers, thereby diminishing the risk of multiple pregnancies [1,2]. Furthermore, freezing and storing of surplus embryos improves the cumulative pregnancy rate per oocyte collection [3–6]. There is, however, evidence suggesting that frozen-thawed embryos have lower developmental competence than fresh embryos [7–10]. The reduced pregnancy rates after frozen embryo transfers can be explained by damage of embryos caused by the freezing and thawing procedures. The loss of blastomeres that occurs in almost half of all thawed embryos is one of the most deleterious consequence of cryopreservation procedure [11]. In addition to blastomere loss, damage of cell membranes [12] and zona pellucida [13,14] has also been found in thawed embryos. The developmental capacity of frozen-thawed embryos can be further impaired by chromosomal defects possibly induced by cryopreservation [15,16]. Therefore, the aim of the current study was to investigate the possibility whether cryopreservation causes chromosomal abnormalities in human preimplantation embryos.

2. Materials and methods

2.1. Patients and embryos

Twenty-eight patients undergoing in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) procedure at the Infertility Clinic of the Family Federation of Finland

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between 1997 and 1999 provided 61 frozen zygotes and cleavage stage embryos for the study after informed consent was obtained from each couple. On average, 2.2 (1–4) embryos were donated by every couple, whereas five couples provided embryos both to study and control group. Twenty-one couples underwent IVF and provided 48 embryos while seven patients underwent ICSI and provided 13 embryos. The details of ovarian stimulation regimen, oocyte collection, IVF and ICSI procedures used in our clinic have been extensively discussed elsewhere [17,18].

2.2. Embryo freezing and thawing

Zygotes and cleavage stage embryos were frozen 20–22 and 44–48 h after insemination/ICSI, respectively. Only the cleavage stage embryos with ≤35% of fragmentation were considered suitable for cryopreservation. Identical freezing program was used for zygotes and cleavage stage embryos. Cryopreservation was accomplished using automated Kryo 10 series II biological freezer (Planer Products Ltd., UK) following a slow freeze protocol using 1,2-propanediol (PROH) (Sigma, USA) as a cryoprotectant [19]. The freezing solution was made-up in phosphate buffered saline (PBS) (Gibco, Life Technologies, UK) supplemented with 20% (v/v) human serum (Finnish Red Cross, Finland). Embryos were first incubated in 1.5 M PROH freezing solution at room temperature (10 min) and then transferred to 1.5 M PROH and 0.2 M sucrose (Sigma, USA) freezing solution. Embryos were thereafter loaded into plastic ministraws (0.25 ml, Pailette Souple, Industrie de la Médecine Vétérinaire (IMV), France) and the freezing program was executed as follows: embryos were placed in the freezing machine at 18.0 °C, cooled at −2.0 °C/min to −8.0 °C, held at −8.0 °C for manual seeding (10 min), cooled at −0.3 °C/min to −30.0 °C, and then at −30.0 °C/min to −150.0 °C, before being plunged into liquid nitrogen.

In thawing, the straws with frozen zygotes and embryos were removed from liquid nitrogen, exposed to air (30 s) and immersed in a water bath at 30 °C (30 s). Zygotes and embryos were incubated in 1.0 M PROH (5 min) and 0.5 M PROH (5 min) in thawing solution (0.2 M sucrose and 20% (v/v) human serum in PBS). Zygotes and embryos were then incubated in thawing solution (10 min) and in sucrose-free thawing solution (10 min).

A total of 61 zygotes and cleavage stage embryos were thawed. Study group ($n = 29$) consisted of 14 zygotes and 15 two-cell embryos. After thawing, study group zygotes and two-cell embryos were cultured in Universal-IVF medium (U-IVF, Medicult, Denmark) up to four- to six-cell stage, after which their chromosomal constitutions were assessed. Control group embryos ($n = 32$) were frozen at four- to six-cell stage, and analysed immediately after thawing. Genetic analysis of control group embryos was performed before resumption of blastomere divisions. Therefore, the number of chromosomes would be unaltered compared to the situation prior to freezing.

2.3. FISH procedure and analysis of results

The chromosomal analysis of embryos was performed applying the FISH method. The reliability of the method with probes for chromosomes 13, 16, 18, 21, X and Y was tested on lymphocyte preparations obtained from healthy males. Embryos were prepared for analysis as follows: zona pellucida was stripped with 0.5% pronase (Sigma, St. Louis, MO, USA); embryos were incubated in Ca^{2+} and Mg^{2+} free medium (Biopsy Medium™, MediCult, Copenhagen, Denmark) (10 min); the blastomeres were separated by gently pipetting the embryos up and down in a thin glass pipette and the individual blastomeres were fixed on poly-L-lysine coated glass slides using 0.01 M HCl + 0.1% Tween-20 [20]. The slides were left to air dry and then dehydrated through an alcohol series. In the first round of hybridisation probes for chromosomes 13 (LSI 13/RB1, Spectrum Green, Vysis, US), 16 (LSI 16/CBFB, mixture Spectrum Red/Green, Vysis, US) and 21 (LSI 21/D21S259, D21S341, D21S342, Spectrum Orange, Vysis, US) were used. The nuclear DNA together with the probes was co-denatured at 70 °C (5 min) and the hybridisation was performed in the moist chamber at 37 °C overnight. The slides were washed at 46 °C in 50% formamide + 2× SSC (3 × 10 min) followed in 2× SSC (10 min) and in 2× SSC + 0.1% Nonidet P-40 (5 min). Subsequently the slides were washed at room temperature in 2× SSC, rinsed in water, air-dried and stained using DAPI (4,6-diamino-2-phenyl-indole) with antifade. The images of nuclei were captured with an Olympus epifluorescence microscope (BX60) equipped with a cooled CCD camera (high sensitive integrating monochrome CCD-camera, JAI CV-M300, Japan) and analysed using ISIS3 software (MetaSystem, Sandhausen, Germany). The recycling was performed as described by Iwarsson et al. [15] using probes for chromosomes 18 (CEP 18, 1:1.5 mixture Spectrum Orange/Green, Vysis, US), X and Y (CEP X, Spectrum Green, LSI SRY, Spectrum Orange, Vysis, US) and applying similar conditions as described for the first hybridisation.

The interpretation of FISH results of blastomeres was carried out according to the recently described scoring criteria [21]. Only totally survived embryos with at least 2/3 blastomeres successfully analysed were included in the study. Both study and control group comprised of embryos with ≤35% of cytoplasmic fragmentation. According to the FISH results embryos were divided into four groups based on the chromosomal constitution of blastomeres: (i) genetically normal embryos, (ii) aneuploid embryos, (iii) mosaic embryos and (iv) chaotic embryos. Mosaic embryos possess genetically abnormal cells along with normal ones while chaotic embryos have only abnormal blastomeres. However, the blastomeres of chaotic embryos have different chromosomal abnormalities.

The comparison of groups was accomplished using χ^2-test and independent-samples Student's t-test. The significance level was considered at $P < 0.05$.

Table 1
Clinical parameters inherent to study and control groups

Clinical parameters	Study group	Control group	*P*
Average women's age ± S.D.[a] (years)	34.2 ± 3.3	34.6 ± 3.7	NS
IVF embryos, *n* (%)	22 (75.9)	26 (81.3)	NS
ICSI embryo, *n* (%)	7 (24.1)	6 (18.7)	
Good quality embryos[b], *n* (%)	14 (48.3)	20 (62.5)	NS
Moderate quality embryos[c], *n* (%)	15 (51.7)	12 (37.5)	

NS: not significant.
[a] S.D.: standard deviation.
[b] Embryos with <20% of fragmentation.
[c] Embryos with 20–35% of fragmentation.

3. Results

The clinical parameters inherent to study and control groups are shown in Table 1. The differences in terms of the women's age, the ratios of IVF and ICSI embryos and the prevalences of embryos with good (<20% fragmentation) and moderate (20–35% fragmentation) quality were not significant between the two groups.

The results of FISH analysis using probes for six chromosomes (13, 16, 18, 21, X and Y) performed on 61 cleavage stage embryos are presented in Table 2. The FISH analysis was successfully accomplished on 259 (85%) of 305 blastomeres. Although there were fewer normal embryos in study (20.7%) than in control (31.3%) group the difference was not statistically significant. The prevalences of aneuploid (10.3% versus 12.5%) and mosaic (44.8% versus 50%) embryos were also similar. However, a higher proportion of chaotic embryos in study (24.1%) compared to control group (6.3%) was observed ($P < 0.05$). Seven (11.4%) of the 61 analysed embryos were aneuploid; three in the study and four in control group. One embryo (1.6%) had trisomy 13, two embryos (3.3%) had trisomy 16, one embryo (1.6%) had monosomy 16, two embryos (3.3%) had trisomy 21 and one embryo (1.6%) had sex chromosome aneuploidy (XYY).

When the rate of chromosomal abnormalities in embryos ($n = 14$) obtained from frozen-thawed zygotes was compared to control group no differences were seen in the proportions of normal (21.5% versus 31.3%), aneuploid (7.1% versus 12.5%) and mosaic (50% versus 50%) embryos. Although the increased level of chaotic embryos was observed among embryos developed from frozen zygotes (21.4%) than in control group (6.3%), the difference was not statistically significant. Embryos ($n = 15$) developed from frozen two-cell embryos and control group embryos had comparable rates of normal (20% versus 31.3%), aneuploid (13.3% versus 12.5%) and mosaic embryos (40% versus 50%). However, markedly higher incidence of chaotic embryos was identified among embryos grown from frozen two-cell embryos (26.7%) than in control group (6.3%).

Table 2
Chromosomal status of study and control group embryos

	Study group	Control group	*P*
n	29	32	
Normal, *n* (%)	6 (20.7)	10 (31.3)	NS
Aneuploid embryos, *n* (%)	3 (10.3)	4 (12.5)	NS
Trisomy 13, *n*	–	1	
Trisomy 16, *n*	–	2	
Monosomy 16, *n*	1	–	
Trisomy 21, *n*	1	1	
XYY, *n*	1	–	
Mosaic embryos, *n* (%)	13 (44.8)	16 (50)	NS
Chaotic embryos, *n* (%)	7 (24.1)	2 (6.3)	<0.05

NS: not significant.

4. Discussion

Objective of the current study was to investigate the effect of cryopreservation on chromosomal abnormalities in human preimplantation embryos. To this end, the chromosomal constitutions of 61 cleavage stage embryos were determined using probes for chromosomes 13, 16, 18, 21, X and Y. The embryos in study group were frozen either at zygote or two-cell stage and were cultured after thawing up to four- to six-cell stage before analysis. Although the embryos in control group were also frozen, they were analysed immediately after thawing. This ensures that the chromosomal constitutions observed in these embryos were unaltered compared to the situation prior to freezing [22]. Thus, the comparison of the proportion of chromosomally abnormal embryos between study and control groups allows to estimate the effect of cryopreservation on the formation of chromosomal defects in human preimplantation embryos.

Our results demonstrated a trend toward a lower number of genetically normal embryos among embryos that had undergone cellular divisions post-thaw (20.7%) than embryos analysed immediately after thawing (31.3%), but the difference was not statistically significant. The comparison of different studies published on chromosomal status of preimplantation embryos is complicated by the varying number and specificity of DNA probes used. Nevertheless, it has been concluded that the prevalence of chromosomal defects in frozen-thawed embryos is higher than in fresh embryos [15,16]. In the study by Iwarsson et al. a high incidence of chromosomal abnormalities for chromosomes 15, 16, 17, 18, X and Y was demonstrated in frozen-thawed human two- to nine-cell embryos after 24 h post-thaw culture [15]. In their study, 25% of the embryos had a normal number of the chromosomes tested. For fresh embryos, Munne et al. showed that 43% of non-cryopreserved embryos were normal for the five chromosomes tested (13, 18, 21, X and Y) [23]. The proportion of genetically normal embryos in control group (31.3%) in our study is comparable to the result of the

study by Bahce et al. [24]. After two rounds of FISH for chromosomes 1, 4, 6, 7, 14, 15, 17, 18 and 22 it was revealed that approximately 1/3 of fresh embryos were chromosomally normal [24].

In the current study, the proportion of chaotic embryos was significantly higher in study (24.1%) than control (6.3%) group. The higher proportions of chromosomally chaotic embryos were observed both among embryos developed from frozen zygotes and two-cell embryos than in control group, but only the latter difference was statistically significant. Our finding of elevated incidence of chromosomally chaotic frozen-thawed embryos is in agreement with previous studies also demonstrating more chaotic embryos among frozen-thawed (15–20%) [15,16] than fresh (6%) embryos [25]. In chaotic embryos blastomeres possess a random chromosomal constitution. This is likely to occur when all dividing cells are unable to properly segregate their chromosomes between daughter blastomeres, for example, because of dysfunctional spindles. It has been suggested that zygotes should be cryopreserved before syngamy [26] because freezing may cause irreversible disruption of the spindle [27] leading to chromosomal abnormalities. Although in the present study freezing of the zygotes was commenced 20–22 h after insemination or ICSI, it cannot be excluded that some zygotes had already passed beyond the optimal time for freezing. The precise timing of freezing is also critical in cryopreservation of cleavage stage embryos as freezing at the time of cellular divisions may disturb the partitioning of chromosomes into the daughter cells [28]. The culprit in this case may also be the detrimental effect of cooling on the integrity of spindles. However, it is common practice to freeze all cleavage stage embryos together on the second or third day after insemination or ICSI. This heterogeneous group is likely to contain embryos invarious stages of blastomere division implying that some of the cleavage stage embryos may be more prone to freezing damage than others.

Our results suggest that cryopreservation may induce chromosomal abnormalities in frozen-thawed human embryos. The higher number of chromosomal defects in embryos that have undergone cellular divisions after thawing could at least partially account for the compromised viability of cryopreserved embryos after uterine transfers. Additional studies involving larger numbers of cryopreserved embryos are still needed before a definite conclusion can be drawn about the potential impact of cryopreservation on the formation of chromosomal abnormalities in preimplantation embryos.

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