Integrity rate of pronuclei after cryopreservation of pronuclear-zygotes as a criteria for subsequent embryo development and pregnancy

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BACKGROUND: The aim of this study was to examine whether the integrity rate of pronuclei, after cryopreservation of pronuclear zygotes, could be a predictor of future embryo development and implantation. METHODS: Two-pronuclei stage zygotes (n = 862) were cryopreserved by aseptic rapid freezing in 15% ethylene glycol + 15% DMSO + 0.2 M sucrose with a 4-step exposure in 12% (v:v), 25, 50 and 100% rapid freezing solution for 2, 1, 1 min and 30–50 s, respectively, at room temperature, and then plunged into liquid nitrogen. Zygotes were rapidly warmed at a speed of 30 000 °C/min and subsequently expelled into a graded series of sucrose solutions (1.0, 0.5, 0.25, 0.12 and 0.06 M) at 2.5 min intervals and in vitro cultured for 5 days. If embryos had developed to blastocysts on the 5th day, they were transferred to the recipients. The rest of the embryos were fixed for evaluation of chromatin. RESULTS: Zygote development, up to the expanded blastocyst stage, after in vitro culture, was 40%, if the integrity rate of pronuclei was high, and 4% if the integrity rate was low (P < 0.05). The pregnancy rate after transfer of the 5-day blastocysts depended on the pronuclear integrity rate: 43% (125 pregnancies after transfer of 291 blastocysts) for those with a high integrity, and only 1 in 5 (20%) for the few blastocysts which had shown a pronuclear low integrity rate (P < 0.05). CONCLUSIONS: Integrity rate of pronuclei after cryopreservation of pronuclear zygotes was a predictor of future embryo development and implantation: high integrity rate resulted in high pregnancy rate, while zygotes with low integrity rate of pronuclei after cryopreservation had low developmental potential.

Keywords: zygotes; cryopreservation; pronuclei; morphology; integrity rate

Introduction

Study of cryopreservation of human pronuclear zygotes is an important topic, as it is illegal to cryopreserve a zygote after fusion of the pronuclei due to ethical difficulties in some countries.

Conventional (slow) freezing of human pronuclear zygotes has been the most widely used method of storage up until now (Siebzehnrübl et al., 1989; Veeck et al., 1993; Van den Abbeel et al., 1997; Al-Hasani et al., 1999; Damario et al., 1999). After transfer of embryos developed from conventionally (slowly) frozen pronuclear zygotes, numerous babies have been born in some centers. However, with the low efficacy of the zygote freezing technique, the ‘frozen zygote’ pregnancy rate is lower than that resulting from fresh pronuclear zygotes. There have also been several reports of the successful cryopreservation of human pronuclear zygotes by direct plunging into liquid nitrogen (so called rapid freezing) (Park et al., 2000; Jelinkova et al., 2002; Liebermann and Tucker, 2002; Liebermann et al., 2002a,b; Selman and El-Danasuri, 2002; Isachenko et al., 2003, 2004a,b, 2005). Rapid freezing as a method of cryopreservation is now the object of intensive investigation in numerous laboratories.

The evaluation of the quality of pronuclear zygotes after thawing and before in vitro culture provides useful information. The integrity rate of pronuclei (i.e. their morphology) can be a criterion of cryostability, developmental potential of the zygote and/or effectiveness of concrete protocol of cryopreservation (conventional freezing as well as rapid freezing). However, data regarding the correlation of the post-thawing integrity rate of pronuclei with the potential for embryos to develop and implant is limited.

The aim of this study was to examine whether the integrity rate of pronuclei after cryopreservation of pronuclear zygotes can be a predictor of future embryo development and implantation.
Materials and Methods

Except where otherwise stated, all chemicals were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). Written informed consent was obtained from the participating couples for the cryopreservation and culture of zygotes. The study was performed in Bulgaria in period from September 2004 to June 2007 and approval was obtained from the State Ethics Committee of Institute of Biology and Immunology of Reproduction. Here, we describe the manipulation of cryopreserved pronuclear zygotes from patients who were not pregnant after transplantation of embryos derived from fresh pronuclear zygotes.

Pronuclear zygote derivation and evaluation of pronuclei

A total of 281 patients ranging in age from 21 to 40 years (median age 33.6) volunteered to have their zygotes cryopreserved by rapid freezing.

Patients were stimulated for IVF–ICSI with triptorelin (Decapeptyl®, Ferring, Kiel, Germany) and recombinant follicle-stimulating hormone (Puregon®, Organon, Oss, The Netherlands) according to the ‘long’ protocol. Ovulation was induced by the administration of 10 000 IU human chorionic gonadotrophin (hCG, Pregnil®, Organon, Oss, The Netherlands). Oocytes were retrieved 34–36 h later and fertilized with the husband’s sperm through conventional IVF or ICSI techniques. Progressive motile sperm for insemination was isolated by ‘swim up’ technique. Conventional IVF was performed routinely 6 h after oocyte collection (Day 0). Motile sperm concentration was 0.5–1.0 × 10^6/ml according to sperm quality. ICSI was performed 4–5 h after oocyte collection. Denudation of oocytes was performed by gentle pipetting after a short incubation in 80 IU/ml hyaluronidase. After sperm micro-injection, the oocytes were placed into culture media individually.

The quality of pronuclei were examined 17–18 h after fertilization. Zygotes were scored on an inverted microscope at a magnification of ×400. Pronuclear morphology (Z-score) of zygotes was performed as described by Scott et al. (2000). Photo documentation was performed for fresh zygotes, zygotes obtained 10 min after thawing, and after 8, 24, 48, 72, 80, 92, 100 and 120 h.

The integrity rate of pronuclei after thawing was distributed into two types: high and low. If after 10 min of thawing it was possible to clearly observe: (i) a border of pronuclear membrane and (ii) at least half of the chromatin in a condensed form (nucleoli), this integrity rate was denoted as high (Fig. 1a–e). If, after 10 min of thawing, it was not possible to clearly observe a structure of these two elements of pronuclei, this integrity rate was denoted as low (see a–d in Figs 2 and 3).

Figure 1: The same two-pronuclear zygotes scored as Z2; development appeared to be dependent on the post-thawing integrity rate of pronuclei (a and b) fresh pronucleus, (c and d) pronucleus after thawing. Embryos after respective times in in vitro culture (e–m). The same 48-h embryo is shown photographed with a different focus, for demonstration of fragmentation (g and h). Five-day blastocyst (m), transferred
The zona pellucida of some embryos (from all patients with age >35 years) was drilled for assisted hatching, using pronase, on the second or 3rd day of the culture (Fong et al., 1998). From each patient, two to three from the 1553 normal zygotes were used for subsequent in vitro culture and transplantation of embryos. The rest of the zygotes \( n = 862 \) were used for cryopreservation, thawing, subsequent culture and transplantation to recipients. All the cryopreserved zygotes after thawing were cultured for an additional 5 days. If the embryos on the 5th day had developed to blastocysts, these blastocysts were transferred to patients. The rest of the zygotes which did not develop to blastocysts were denoted as dead and fixed in Hoechst 33342, for evaluation of chromatin.

**Rapid freezing, thawing and culture of zygotes**

Two-pronuclei stage zygotes \( n = 862 \) were cryopreserved by a modified version of the aseptic rapid freezing technique by (Isachenko et al., 2007). Rapid freezing solution was composed of Leibovitz L-15 medium supplemented 10% Serum Substitute Supplement (Irvine Scientific, Santa Ana, CA, USA), 15% ethylene glycol, 15% DMSO and 0.2 M sucrose. Pre-cooling treatment (exposure in cryoprotectants) was performed by step-wise exposure in 12% (v:v), 25, 50 and 100% of the rapid freezing solutions for 2, 1, 1 min and 30–50 s, before plunging into liquid nitrogen, respectively. Cooling and thawing of embryos was performed in Cut Standard Straws (CSS) (Isachenko et al., 2007). CSS was produced from standard insemination 0.25 ml straws (CSS, Medical Technology GmbH, Altdorf, Germany) which were cut at an angle approximately 45°. Rapid freezing medium with one or two zygotes was dropped into this cut part of the straw. After exposure in rapid freezing medium, one or two zygotes with 0.75 μl of this medium was aspirated to the tip of pipettor and transferred to CSS. The CSS were first loaded into 0.5 ml straws, which were closed at both sides using an ultrasound sealer (Medical Technology GmbH, Altdorf, Germany), and plunged into liquid nitrogen with a cooling speed of 600°C/min.

For rapid thawing, the solution composed of Leibovitz L-15 medium, 10% Serum Substitute Supplement, 1.0 M sucrose and 5% polysaccharide polyvinyl piroldione with a molecular weight 10 kDa (PVP-10) was used. CSS were first removed from the 0.5 ml straws, which were still half submerged in liquid nitrogen, prior to plunging them into sucrose solution. This mode allowed for the simultaneous removal of cryoprotectant and rapid thawing. Zygotes

![Figure 2: The same two-pronuclear zygotes scored as Z3: development appeared to be dependent on the post-thawing integrity rate of pronuclei (a and b) fresh pronucleus, (c and d) pronucleus after thawing. Embryos after respective times in vitro culture (e–m). Embryo stained by Hoechst 33342 (m). Note: immune fluorescence staining of embryo shows normal cleavage and absence of a nuclear chromatin deformation.](image-url)
were rapidly warmed at a speed of 30,000°C/min. They were subsequently expelled into a graded series of sucrose solutions (1.0, 0.5, 0.25, 0.12 and 0.06 M) at 3 min (in 1.0 M sucrose) and 2.5 min (in rest of solutions) intervals.

After thawing and washing, zygotes were located to the same medium, in which they were cultured before rapid freezing.

After thawing, all zygotes were cultured for 5 days in 5% CO₂ plus air at 37°C in Blast-Assist Medium (I and II) (Medicult, Denmark) according to a standard protocol.

In accordance with the appearance of the pronuclei 10 min after thawing, the zygotes were distributed into two groups: zygotes with low integrity rate of pronuclei and zygotes with high integrity rate of pronuclei.

**Embryo transfer and pregnancy**

Embryo transfer (only one blastocyst per one patient) was performed on Day 5 if the embryo was evaluated to be morphologically normal.

Pregnancy was defined when an increase in serum hCG values (>20 IU/l) was measured 11 and 13–15 days after embryo transfer. Clinical pregnancy was considered established if a fetal sac was visualized on an ultra-sound at gestational weeks seven to eight.

**Statistical analysis**

Pronuclei post-thawing integrity rate on the parameters assessed were evaluated by ANOVA. Various characteristics were summarized by mean and SD within groups.

The level of statistical significance was set at a *P* < 0.05.

**Results**

In Figs 1, 2 and 3, different cases of the relationship between the pronuclei post-thawing integrity rate and following development of zygotes are presented. Good pronuclear integrity rate resulted in the development of embryos to blastocysts with a high implantation rate after transfer (Fig. 1).

Development of some zygotes with a low post-thawing pronuclear integrity rate was normal to a late stage (compacted morula or early blastocysts). However, later an arrest of cleavage was observed (Fig. 2). Typical developmental defectiveness of embryos after a low post-thawing pronuclear integrity rate was also observed (Fig. 3).
Immune fluorescence staining of some embryos, which were not developed to blastocysts, showed normal cleavage and absence of a nuclear chromatin deformation (Fig. 2). In contrast, staining of other embryos (generally with intensive vacuolization of cytoplasm) showed defective cleavage and deformation of nuclear chromatin (Fig. 3).

Fresh pronuclear zygotes scored by Scott et al. (2000) were distributed into grades Z1, Z2, Z3 and Z4 as follows: 6, 37, 47 and 10%, respectively (Fig. 4). A high post-thawing integrity rate was detected in 85% and a low integrity rate was detected in 15% of the zygotes (Fig. 4). It was shown that there was an association between the scoring group and the post-thawing pronuclear integrity rate (Fig. 4). Approximately one zygote in seven and one zygote in eight, from the Z2 and Z3 scoring groups, respectively, had low post-thawing integrity rates. In contrast, in about half of the zygotes from the Z1 group, the integrity rate was low. A mixed cryostability was shown in zygotes from the Z4 group: one zygote in five had a low post-thawing integrity rate of pronuclei (Fig. 4).

Zygote developmental rates up to the expanded blastocyst stage, after in vitro culture, were 40% (291 from 732 zygotes) if the integrity rate of pronuclei was high, and 4% (5 from 130) if the integrity rate was low ($P < 0.05$) (Fig. 5).

After transplantation of 296 five-day blastocysts to 296 recipients, 126 patients became pregnant. Pregnancy rates appeared to depend on the high or low pronuclear integrity rates after cryopreservation; 43% of blastocysts which had high integrity rates produced pregnancies while only one group, respectively, had low post-thawing integrity rates. In contrast, in about half of the zygotes from the Z1 group, the integrity rate was low. A mixed cryostability was shown in zygotes from the Z4 group: one zygote in five had a low post-thawing integrity rate of pronuclei (Fig. 4).

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pregnancy resulted from the five blastocysts (20%) which had shown low pronuclear integrity rates \((P < 0.05)\) (Fig. 6). All born babies are healthy.

**Discussion**

The protocol of cryopreservation by direct plunging into liquid nitrogen was used for pronuclear zygotes. For this protocol, the terminology ‘vitrification’ is often used. However, at the relatively slow speed of cooling (–600\(^\circ\)C/min) an ice-free state of vitrification medium is not maintained through cooling and warming. The glass-formation at cooling and the stability of the amorphous state of solution at warming have been previously reported (Baudot et al., 2000). It was established, that even 40% DMSO, which is a better glass former than ethylene glycol, has a critical cooling rate, to avoid ice formation, of 500\(^\circ\)C/min, and a calculated critical warming rate, to avoid ice formation (devitrification), of over one billion degrees per minute (Baudot et al., 2000). In the described solution, the carrier medium and serum solutes provide additional stability against ice formation, but 30% penetrating cryoprotectants are not able to prevent ice forming during the cooling, and especially described warming, conditions. This explains why our experiments are not ‘real’ vitrification. By this reason, we use terminology ‘rapid freezing’ instead ‘vitrification’. For the same reason, we use the terminology ‘thawing’ instead ‘warming’.

The integrity rate of pronuclei after thawing was distributed as two types: high and low. The appearance of two elements of pronuclei, their membrane and nucleoli, were taken into account. In the planning of our experiments, we were going to determine a post-thawing integrity rate of these two elements separately, with distribution of all zygotes into four groups: (i) high integrity rate for both pronuclear membranes and nucleoli, (ii) high integrity rate of pronuclear membranes and a low rate for nucleoli, (iii) low integrity rate for pronuclear membranes and a high one of nucleoli and (iv) low integrity rate of both pronuclear membranes and nucleoli.

However, later, the analysis of photographs had evidenced that it was not necessary to evaluate these two elements separately. If the integrity rate of a nucleus membrane was high, the rate for at least half of the nuclei was also high. Soon after thawing, the integrity rate of the pronuclear membrane was high, but the appearance of pronuclei was not clear. However, after some time the re-condensation of chromatin took place and 10 min after thawing and washing, the appearance of the nucleoli became clear. We have described this phenomenon of post-thawing nucleoli dispersion following re-condensation earlier (Isachenko et al., 2005).

There is a direct relationship between the post-thawing integrity rate of the pronuclear membrane and nucleoli. This fact allows us to form two groups, taking into account only the post-thawing integrity rate of both nucleolar membranes and nucleoli together: high and low integrity rates.

Established regularity of the integrity rate of pronuclei after thawing of zygotes can be a predictor of future development, and can play a role in the practice of assisted reproductive technology. The study protocol of a number of laboratories presupposes the transfer of a single blastocyst. If after thawing of one or two pronuclear zygotes and 5 days of *in vitro* culture of these zygotes to the blastocyst stage, these zygotes do not demonstrate a development to blastocysts, the transfer of these embryos is probably pointless.

After thawing of zygotes, we suggest to take into account the integrity rate of pronuclei. If this rate is high, at least in one zygote, these zygotes can be *in vitro* cultured for 5 days and embryos can be transferred. If the integrity rate of pronuclei after thawing is low, it is necessary to thaw other zygotes,

![Figure 6: Pregnancy rate after transfer of blastocysts dependent on quality of pronuclei (Z-scoring) and their post-thawing integrity rate.](image-url)
because the developmental prognosis of the first part of zygotes is negligible.

The initial fresh zygote score does not seem to have a clear impact both on either the developmental or implantation ability after freezing and thawing, compared to the recovery itself of integrity for whatever figure of pronuclei. Post-warming pronuclear integrity rate refers more generally to cellular integrity. However, it is possible that the use of the more detailed zygote scoring will allow one to search for a strict connection between the scoring of zygotes and cryostability of pronuclei. For example, it has been established that a cumulated pronuclear score could be used as a single prognostic tool for implantation of both fresh and frozen–thawed zygotes (Senn et al., 2006).

Osmosis plays a central role throughout all negative effects of the cryopreservation process of human pronuclear embryos (Isachenko et al., 2004b). In contrast with zygotes of animal species, human pronuclear zygotes that are vitrified, warmed and directly rehydrated with intense osmotic processes, are fully destroyed. Taking into account the fact that the saturation by cryoprotectants is also accompanied by osmotic processes, our aseptic technology includes a step-wise method of saturation by cryoprotectants (Isachenko et al., 2005).

The results of this investigation show that membranes of nucleoli are more sensitive to osmotic stress than are the nucleoli themselves, and the integrity rate of these membranes must be the most informative criteria for the integrity rate of pronuclear human embryos after cryopreservation.

When analysing the photographs of cryopreserved pronuclear zygotes and developing embryos, we did try to search for another prognostic criteria for cryopreserved pronuclear zygotes and connect the post-thawing integrity rate of these zygotes and their following development. The appearance of blastomere fragmentations and vacuolization of developing embryos were analysed. By our supposition, appearance of fragmentation was an ‘autonomic’ process and it was not connected with cryostability of zygotes. In zygotes with a high integrity rate of pronuclei after thawing and good developmental rate to blastocysts, enough intensive fragmentation was observed (Fig. 1f–i). In contrast with fragmentation, vacuolization was a detector of the low developmental potential of cryopreserved pronuclear zygotes. Morphology of the developing embryos, in Figs 2 and 3, in 72 h of culture, seemed good (e–h). Later, however, the appearance of vacuoles (Figs 2 and 3, i) predicted defective development.

It was established that deformation of chromat in was not connected directly with defective cleavage of embryos, as reflected in a failure to develop to a blastocysts stage. For example, some zygotes with a low post-thawing integrity rate were developed to compacted morula (Fig. 2) or early blastocyst and then stopped developing. Serious chromat abnormalities in such embryos were not detected. In contrast, deformation of chromatin in embryos with intensive vacuolization was detected (Fig. 3). At the same time, it was noted, that in both cases of degenerative development of the followed embryos, the post-warming pronuclear integrity rate of zygotes was low.

In conclusion, the integrity rate of pronuclei after cryopreservation of pronuclear zygotes is a predictor of future embryo development and implantation, i.e. a high integrity rate results in a high pregnancy rate, and zygotes with a low integrity rate of pronuclei after cryopreservation have a low developmental potential.

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References


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