Sucrose concentration influences the rate of human oocytes with normal spindle and chromosome configurations after slow-cooling cryopreservation*

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BACKGROUND: Recently described slow-cooling cryopreservation protocols involving elevated sucrose concentrations have improved survival frequencies of human oocytes, potentially overcoming a major hurdle that has limited the adoption of oocyte storage. Because implantation rates of embryos from frozen oocytes remain generally low, it is still debated whether, irrespective of survival rates, this form of cryopreservation leads inevitably to the disruption or complete loss of the metaphase II (MII) spindle. METHODS: Human oocytes with an extruded polar body I (PBI) were cryopreserved using a slow-cooling method including 1.5 mol/l propane-1,2-diol (PrOH) and alternative sucrose concentrations (either 0.1 or 0.3 mol/l) in the freezing solution. Fresh control and frozen-thawed survived oocytes were analysed by confocal microscopy to evaluate MII spindle and chromosome organizations. RESULTS: Of the 104 oocytes included in the unfrozen group, 76 (73.1%) displayed normal bipolar spindles with equatorially aligned chromosomes. Spindle and chromatin organizations were significantly affected (50.8%) after cryopreservation involving lower sucrose concentration (61 oocytes), whereas these parameters were unchanged (69.7%) using the 0.3 mol/l sucrose protocol (152 oocytes). CONCLUSIONS: Partial disruption of the MII spindle and associated chromosomes accompanies inadequate cryopreservation during slow cooling. However, protocols adopting higher sucrose concentration in the freezing solution promote the retention of an intact chromosome segregation apparatus comparable in incidence to freshly collected oocytes.

Key words: oocyte cryopreservation/slow cooling/spindle apparatus/chromosomes/sucrose

Introduction

Oocyte cryopreservation has been a much anticipated advance in human IVF and reproductive sciences that may extend female reproductive potential, while avoiding ethical and legal complications originating from the storage of embryos and fertilized oocytes. Because human oocytes are particularly sensitive to cryopreservation, irrespective of the employment of either slow (controlled) cooling or vitrification as cryopreservation methods, this prospect has not been realized. During slow cooling, the sources of cell damage are varied. Intracellular ice formation (IIF), cryoprotectant (CPA) toxicity, osmotic stress, non-physiological supra-zero temperatures, pH instability and generation of reactive oxygen species (ROS) are factors believed to affect oocyte viability (Fuller and Paynter, 2004). Several oocyte organelles are suspected targets for cryodamage. Disruption or complete loss of the metaphase II (MII) spindle is likely to compromise oocyte functionality. The importance of this structure is well known, subserving not only chromatid segregation at meiosis II but also the fertilization process (Winston et al., 1995) and establishment of the cell polarity (Albertini and Barrett, 2004). As a highly dynamic structure originating from the reversible polymerization of tubulin subunits, across mammalian species the oocyte MII spindle appears to be prone to damage from non-physiological depolymerization as a consequence to its extreme susceptibility to various extrinsic factors, especially temperature. In the human, the MII spindle has been found to disassemble irreversibly in response to very short exposure to 0°C (Zenzes et al., 2001) or even transient culture at a slightly lower, suboptimal temperature (Pickering et al., 1990). This has originated...
the belief that damage to this structure is highly likely, if not inevitable, as a result of the temperature changes involved in cryostorage. In effect, Boiso et al. (2002) reported that chromosome and spindle organizations are extensively affected by slow cooling. However, this matter remains highly controversial, because other authors have disputed such conclusions, suggesting that slow cooling does not compromise the MII spindle and chromosome organizations (Gook et al., 1993; Stachekki et al., 2004). Such a conflicting evidence may have been generated in some cases by suboptimal quality of the material examined, or inappropriate methodologies. Clinical data are generally inadequate or too scarce to contribute to shed some light on the possible consequences of cryopreservation on chromosome segregation at meiosis II. Considering that chromosome anomalies are known to have developmental consequences, an assessment of the comparative ability of embryos from fresh or frozen oocytes to contribute to test this hypothesis, but unfortunately the studies conducted so far have not been designed with such an intent.

Irrespective of the increasing number of reports of pregnancies from cryopreserved oocytes (Borini et al., 2004; Borini et al., 2005; Chen et al., 2005; Levi Setti et al., 2006), because the safety of oocyte cryopreservation remains an unresolved concern, with this study we aimed at establishing the possible impact on the MII spindle and chromosome configurations of two alternative slow-cooling protocols differing in the concentration of the non-penetrating CPA sucrose in the freezing solution. In a previous study (Fabbri et al., 2001), it was found that increasing the sucrose concentration from 0.1 to 0.3 mol/l dramatically improves the survival rate of human oocytes from 40 to about 80%. However, concerns have been raised that an increase in CPA concentration, and hence in the extent of osmotic stress to which the oocyte is exposed (Paynter et al., 2005), may jeopardize viability, with possible, important consequences on the stability of the MII spindle.

Materials and methods

Source of oocytes

This study was approved by the Public Health Agency of the Italian government, National Health Institute and the Institutional Review Boards of the participating clinics. Oocytes were obtained from consenting couples undergoing assisted reproduction treatment from January 2004 to April 2005. Only oocytes donated from women younger than 36 years (mean ± SD: 32.6 ± 2.9) were used. Controlled ovarian stimulation was induced with long protocols using GnRH agonist and rFSH, according to the standard clinical protocols routinely employed by the participating clinics. HCG (10 000 IU) was administered 36 h before oocyte collection. After retrieval, oocytes were cultured in medium used for standard IVF for 3–4 h. Complete removal of cumulus mass and corona cells was performed enzymatically using hyaluronidase (20–40 IU/ml) and mechanically by using fine-bore glass pipettes. Only oocytes devoid of any sort of dysmorphisms, showing an extruded polar body I (PBI) thus presumably at the MII stage, were assigned to the control or study groups. According to their assignment, they were either fixed or frozen after a total period of time of about 4 h following retrieval.

Cryopreservation solutions

Oocytes were cryopreserved using a slow-cooling method. All cryopreservation solutions were prepared using Dulbecco’s phosphate-buffered solution (PBS) (Gibco, Life Technologies Ltd, Paisley, UK) and a plasma protein supplement (PPS) (10 mg/ml), final concentration (Baxter AG, Vienna, Austria). The freezing solutions were 1.5 mol/l propane-1,2-diol (PrOH) + 20% PPS in PBS (equilibration solution) and 1.5 mol/l PrOH + either (a) 0.1 or (b) 0.3 mol/l sucrose + 20% PPS in PBS (loading solutions), as described by Fabbri et al. (2001). The thawing solutions were (i) 1.0 mol/l PrOH + sucrose + 20% PPS, (ii) 0.5 mol/l PrOH + sucrose + 20% PPS and (iii) sucrose + 20% PPS. Sucrose concentration in the thawing solutions was 0.2 and 0.3 mol/l, depending of whether loading solutions (a) or (b) had been used for freezing, respectively.

Freezing procedure

Oocytes were washed in PBS supplemented with 20% PPS. One or two oocytes were subsequently placed in the equilibration solution containing 1.5 mol/l PrOH + 20% PPS for 10 min. Afterwards, oocytes were transferred to the solution containing 1.5 mol/l PrOH + 0.1 or 0.3 mol/l sucrose + 20% PPS, at room temperature (RT) for 5 min, then loaded into plastic straws (Paillettes Crystal 133 mm; Cryo Bio System, France) and placed into an automated Kryo 10 series III biological freezer (Planer Kryo 10/1.7 GB). Temperature was gradually lowered from 20 to 8°C at a rate of 2°C/min. Manual seeding was induced during the 10-min holding ramp at −8°C. The temperature was decreased then to −30°C at a rate of 0.3°C/min and finally rapidly to −150°C at a rate of 50°C/min. The straws were finally plunged into liquid nitrogen and stored for later use.

Thawing procedure

The straws were rapidly air-warmed for 30 s and then plunged into a 30°C water bath for 40 s. The CPA was removed at RT by step-wise dilution. Oocytes were expelled in the first thawing solution, 1.0 mol/l PrOH + sucrose + 20% PPS for 5 min, then equilibrated in 0.5 mol/l PrOH + sucrose + 20% PPS for additional 5 min. Finally, they were placed in sucrose + 20% PPS for 10 min before final dilution in PBS + 20% PPS for 20 min (10 min at RT and 10 min at 37°C). Before fixation, thawed oocytes were cultured for 3 h in 20 μl of drops of glucose-free medium normally used for embryo culture under warm mineral oil at 37°C in an atmosphere of 5% CO₂ in air.

Immunofluorescence

Only morphologically normal oocytes were used for this study. After freezing and thawing, oocytes showing swelling or shrinkage, vacuoles, membrane blebbing or other anomalies were not considered viable and suitable for microscopy analysis. Oocytes were fixed and processed for microtubules and chromatin immunofluorescence as previously described (Combelles et al., 2002).

Fixation and extraction was carried out for 30–60 min at 37°C in a microtubule-stabilizing buffer containing 100 mM PIPES, 5 mM MgCl₂, 2.5 mM EGTA, 2% formaldehyde, 0.1% Triton-X-100, 1 mM taxol, 10 μM of aprotinin and 50% deuterium oxide (Cekleniak et al., 2001). Washing and storage was conducted with a blocking solution of PBS supplemented with 2% bovine serum albumin, 2% powdered milk, 2% normal goat serum, 100 mM glycine and 0.01% Triton-X-100 containing 0.2% sodium azide. After storage in blocking solution (for a few weeks maximum), oocytes were further processed for immunostaining, through serial incubations with primary and secondary antibodies (1 h per antibody at 37°C with shaking) followed by washing (three washes of 15 min) in blocking solution after each antibody incubation. Microtubule staining was performed by...
using a mouse monoclonal anti-α/β tubulin antibody mixture (Sigma Biosciences, Milan, Italy) diluted 1:150 in wash solution, followed by treatment with a 1:50 dilution of an affinity-purified fluorescein-labelled donkey anti-mouse IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA). Chromatin staining was obtained by using either propidium iodide or Hoechst 33258 (30 min), whereas mounting was carried out with a mixture of 50% glycerol and 25 mg/ml of the antifade agent sodium azide. Oocytes were analysed using either a fluorescence microscope (Axioplan 2, Zeiss; ×40 objective; digital images collected with a Leica DFC350 FX camera interfaced with IM500 Leica software) or a IX81 Olympus confocal microscope (×60 objective; digital images collected with Fluoview FW500).

Statistical analysis
Statistical comparison between fresh and frozen groups was carried out on pooled data using chi-square analysis. A P-value <0.05 was considered significant.

Results
Three hundred and seventeen PBI oocytes were successfully examined through confocal microscopy for the assessment of the MII spindle and chromosome organizations. The survival rates of the cryopreserved oocytes with either the 0.1 or the 0.3 mol/l sucrose protocol were 35 and 74%, respectively, an outcome comparable to those published in previous articles (Fabbri et al., 2001; Borini et al., 2004; Borini et al., 2005; Chen et al., 2005; Levi Setti et al., 2006). The loss of viability was observed in the large majority of cases (>80%) immediately after thawing or during rehydration, although it was rarely observed during the 3 h of post-thaw culture.

Spindle organization was considered morphologically normal when a dense, bipolar (barrel-shaped or elliptical) array of microtubules was observed. Chromosome distribution was regarded regular when sister chromatids exhibited metaphase alignment on the equatorial plane of a symmetric spindle. Basically, three different classes of spindle and chromosome configurations were observed using these criteria to define deviations from the commonly observed MII phenotype (Battaglia et al., 1996; Hodges et al., 2002; Combelles et al., 2003; Hunt et al., 2003): (i) bipolar spindle, aligned chromosomes; (ii) disarranged spindle, non-aligned chromosomes and (iii) absent spindle, non-aligned or dispersed chromosomes (Figure 1).

Of the 104 oocytes included in the unfrozen control, 76 (73.1%) displayed normal (bipolar) spindle and chromosome configurations, 18 (17.3%) showed anomalies involving both the spindle and the chromosomal arrays and 10 (9.6%) appeared devoid of organized microtubules, although possessing non-aligned or dispersed chromosomes (Table I). In the oocytes frozen with the 0.1 mol/l sucrose protocol (n = 61), altered frequencies were found in the three classification categories. A bipolar spindle associated to an orderly array of chromosomes was observed in only 31 (50.8%) of oocytes. Compared to the fresh control, such a difference was found to be statistically significant (P < 0.05). In the same treatment group, 26 oocytes (42.6%; P < 0.05) were classified as having non-bipolar or grossly abnormal spindles with disarranged chromosomes, and four (6.6%) exhibited only disarranged or dispersed chromosomes without microtubule staining. In the treatment group corresponding to the 0.3 mol/l sucrose protocol, oocytes (n = 152) were classified according to the categories (i), (ii) and (iii) with frequencies comparable to those of the control group. In particular, regular spindle and chromosome arrangement was ascertained in 106 (69.7%; P = NS) oocytes, whereas the disruption or total loss of spindle microtubules was detected in 35 (23.0%) and 11 (7.3%) oocytes, respectively.

Discussion
Initial attempts at adopting oocyte cryopreservation for storing the reproductive potential of IVF patients date back as early as 1986 (Chen, 1986; Al-Hasani et al., 1987), but current cryopreservation techniques remain unable to generate clinical results consistently comparable to those achievable with embryo freezing. Protocols used lately have improved survival frequencies (Fabbri et al., 2001; Boldt et al., 2003), thereby overcoming a major hurdle that limited widespread utilization of oocyte storage. Despite promising advances, it remains unclear why cryopreserved oocytes can survive, fertilize, cleave with high rates and yet implant with rates that appear compromised (Borini et al., 2005; Levi Setti et al., 2006). It has been suggested repeatedly that the loss of functionality of frozen oocytes may be secondary to the meiotic spindle sensitivity to the processes of freezing and thawing, a circumstance that could cause increased aneuploidy rates in the embryos thereby generated. The data illustrated in this study indicate that, in the oocytes that are found functional after thawing with a protocol including a high concentration of the non-penetrating CPA in the freezing solution, the meiotic spindle organization and the alignment of associated chromosomes are unaltered. However, our methodology did not allow us to establish whether these structures are affected in the cryopreserved oocytes that do not survive. Our observations also suggest that, to a certain extent, a specific protocol characteristic, i.e. low sucrose concentration, can compromise the chromosome segregation apparatus.

It is not surprising that cytoskeletal damage has been assumed to be an obvious consequence of cryopreservation, considering the detrimental effects on the MII spindle of transient exposure to lower, suboptimal temperatures (Pickering

Figure 1. Representative confocal pictures (×60 magnification) and respective schematic descriptions of bipolar spindle and equatorial chromosome alignment (left), disarranged spindle and irregular chromosome distribution (centre) and absent spindle and disorganized chromosome distribution (right).
et al., 1990; Wang et al., 2001). Specific studies on cryopreserved oocytes, on the contrary, have not been always consistent with this evidence. Using the classical PrOH-based slow-cooling method (Lassalle et al., 1985), Gook et al. (1993, 1994) were the first to challenge the prevailing dogma, reporting that freezing with the conventional slow-cooling protocol does not reduce, in a statistically significant fashion, the rate of oocytes with a normal spindle, nor does it cause aneuploidy after fertilization. Cobo et al. (2001) have further strengthened the hypothesis that chromosome segregation is unaffected in frozen oocytes. Using probes for chromosomes 13, 18, 21, X and Y, these authors conducted a PGD assessment, ascertaining that embryos developed from oocytes frozen with a 1.5 mol/l PrOH and 0.2 mol/l sucrose protocol had a rate of aneuploidy similar to the one of embryos from fresh oocytes (28 and 26%, respectively). However, it should be noticed that only 21 embryos from frozen oocytes were examined in this study. Reassuring evidence has been recently described also by Stachecki et al. (2004), whose data suggest that the proportion of oocytes with barrel-shaped spindles and chromosomes regularly aligned on the spindle equatorial plane is unchanged after cryopreservation (76.7 and 76.7% versus 69.7 and 71.2%, in fresh and frozen oocytes, respectively). It is interesting to note that these authors used a slow-cooling protocol involving the replacement of sodium with the less toxic cation choline, to reduce the so-called solution effect occurring during freezing. Also, it is not clear whether the authors’ choice of exposing oocytes to freezing solutions for varying times (10–20 min or 5–10 min, depending of the freezing solution) may have influenced the outcome of this study. In effect, we have recently shown that exposure times to CPAs may significantly influence the rate of dehydration (Paynter et al., 2005), with possible implications for the risk of IIF. The issue of the quality of oocytes assigned to experiments on cryopreservation is of critical importance. As an example, it has become progressively clearer that inappropriate in vitro conditions have major implications for many oocyte attributes, including the structure and possibly the function of the meiotic spindle (Cekleniak et al., 2001; Sanfins et al., 2003). Therefore, we reckon that oocytes matured in vitro cannot be considered a suitable model for establishing the response of mature oocytes to cryopreservation. Furthermore, polarized light microscopy provides evidence that human oocytes with an extruded PBI may not have reached full meiotic maturation, with their microtubular apparatus still not organized in a properly formed MI spindle (De Santis et al., 2005). Moreover, the same microscopy technique has ascertained that spindle presence is less frequent in oocytes showing the PBI observed at <38 h following HCG injection (Cohen et al., 2004). Importantly, in our study, we observed morphologically normal, supernumerary oocytes that would otherwise have been used for treatment and that were fixed at appropriate times from oocyte recovery and thawing.

Our data indicate that normal MII spindle configuration and chromosome alignment are not disturbed by the processes of freezing and thawing during the application of a modified version of the slow-cooling method including 0.3 mol/l sucrose in the freezing solution. In control oocytes, we ascertained tubulin staining in about 90% of oocytes, although only in a smaller fraction (73.1% of the total) we observed an orderly array of microtubules and a regular chromosome distribution. These figures are comparable to the previously cited findings of Stachecki et al. (2004). It seems established, then, that about 30% of fresh oocytes are found with disorganized microtubules or, in extreme case (5–10%), without a visible spindle. The fraction of oocytes with abnormal spindles varies depending on age (Battaglia et al., 1996), a circumstance that suggested us to limit our observations to oocytes donated by patients younger than 36 years. In our experience, cryopreservation with a slow-cooling method in which sucrose concentration in the loading solution was 0.3 mol/l did not essentially affect the regularity of spindle and chromosome arrangement in the oocytes that maintained a normal morphology after freezing and thawing. This finding confirms and completes previous observations that had documented via polarized light analysis the presence of the MII spindle in oocytes stored with the same protocol (Bianchi et al., 2005). It is difficult to speculate on the reason why transient exposure to just a few degrees below optimal temperature can disrupt irreversibly the spindle, although, during cryopreservation, exposure to much lower temperatures does not cause the same effect. It is possible that permeating CPAs, irrespective of their action of dehydration and mitigation of the solution effect, can influence microtubular polymerization (Johnson and Pickering, 1987). By contrast, the application of the protocol involving a lower sucrose concentration (0.1 mol/l) was associated to a limited but statistically significant reduction in the rate (50.8%) of oocytes with normal spindle and chromosome configurations. Sucrose is a non-penetrating CPA whose concentration in the freezing solution influences the magnitude of the osmotic gradient that draws water from the oocyte. The effect of non-penetrating CPA concentration (0.1 versus 0.3 mol/l) on the rate of dehydration in the pre-cooling phase of exposure to the freezing solution has been described in detail (Paynter et al., 2001; Paynter et al., 2005). Because during slow cooling the amount of residual

<table>
<thead>
<tr>
<th>Treatment (number of oocytes)</th>
<th>Bipolar spindle and aligned chromosomes (%)</th>
<th>Disarranged spindle, non-aligned chromosomes (%)</th>
<th>Absent spindle, non-aligned chromosomes (%)</th>
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</thead>
<tbody>
<tr>
<td>Fresh (104)</td>
<td>76 (73.1)(^a)</td>
<td>18 (17.3)(^a)</td>
<td>10 (9.6)</td>
</tr>
<tr>
<td>Frozen, 0.1 mol/l sucrose (61)</td>
<td>31 (50.8)(^b)</td>
<td>26 (42.6)(^b)</td>
<td>4 (6.6)</td>
</tr>
<tr>
<td>Frozen, 0.3 mol/l sucrose (152)</td>
<td>106 (69.7)(^c)</td>
<td>35 (23.0)(^c)</td>
<td>11 (7.3)</td>
</tr>
</tbody>
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\(^a\) versus \(^b\): \(P < 0.05\) within each column.
water in the intracellular environment is associated to the risk of mechanical damage caused by IIF, it is tempting to speculate that the 0.1 mol/l sucrose protocol is inadequate to ensure sufficient rates of dehydration. Consequently, the MII spindle may be more likely to be disrupted by ice crystals. Conversely, with the 0.3 mol/l sucrose protocol, it may be possible to reach the levels of dehydration that prevent the formation of significant intracellular ice, leaving the spindle relatively undisturbed. Irrespective of IIF during cooling, the MII spindle may be affected during other phases of the cryopreservation process. When oocytes are stored with the 0.1 mol/l sucrose protocol, immediately after removal from liquid nitrogen and rewarmed at room temperature, the spindle, which is initially found in a reduced but significant proportion of oocytes, undergoes depolymerization during rehydration and CPA removal, for reappearing after the completion of the thawing process and incubation for 3 h (Rienzi et al., 2004; Bianchi et al., 2005). It is plausible to suppose, therefore, that the irreversible loss of spindle organization may occur also during this final phase of rehydration after recovery from low temperature storage, although it is not obvious how inappropriate thawing and CPA extraction conditions can influence spindle dynamics.

It is interesting to note that the different ability of the two protocols tested in our study to preserve the microtubular apparatus and associated chromosomal array is consistent with clinical evidence obtained through the application of the same procedures. In particular, fertilization rates in microinjected oocytes stored with the 0.1 mol/l sucrose protocol rarely exceed 45–50% (Tucker et al., 1998; Borini et al., 2004), whereas in oocytes cryopreserved with the alternative protocol these rates (65–75%) are indistinguishable from normal values (Borini et al., 2005; Chen et al., 2005; Levi Setti et al., 2006). The lower incidence of normal spindle organization after freezing with the 0.1 mol/l sucrose protocol may play a causal role in the establishment of such a difference in fertilization rate, in the light of the fact that the meiotic spindle, in addition to co-ordinating chromosome segregation, is also critical for the regulation of meiosis. In fact, in mouse oocytes, alterations (Brunet et al., 2003) or complete loss (Winston et al., 1995) of the spindle have been shown to prevent chromosome segregation and/or exit from meiotic M-phase. The frequency of frozen oocytes with normal meiotic spindle could also have an influence on the chances that clinical pregnancies can evolve to full term. Some oocytes with abnormal spindles could undergo fertilization and generate aneuploid pregnancies. Considering our data, it is interesting to observe that cryopreservation with either the 0.1 or the 0.3 mol/l sucrose protocols has given rise to different abortion rates (20.0 and 14.2%, respectively) (Borini et al., 2004; Borini et al., 2005), but presently it is not possible to draw definite conclusions because of the limited numerical significance of the clinical experiences and the lack of information on the causes of the developmental failures described in these studies.

We reckon that our data represent a significant contribution to the assessment of oocyte quality following cryopreservation, especially with reference to a protocol (0.3 mol/l sucrose) that has already proven to generate high survival rates and that is being adopted for clinical purposes, especially in Italy where embryo freezing is prohibited. However, assessment of the chromosome segregation apparatus cannot be considered exhaustive to the aim of evaluating oocyte viability. This opinion is confirmed by the observation that oocytes cryopreserved with the 0.3 mol/l sucrose protocol, although possessing unaffected spindle and chromosome organizations, develop into embryos that implant with frequencies that have not exceeded 5.7% in at least two independent studies (Borini et al., 2005; Levi Setti et al., 2006). Clearly, other factors other than damage to the cytoskeletal apparatus may be responsible for the poor developmental performance of these oocytes. We are currently involved in investigations using other criteria to evaluate oocyte post-thaw quality. In effect, ultrastructural analysis of oocytes stored with the 0.3 mol/l sucrose protocol has revealed abnormalities of the cytoplasm that may be interpreted as a possible cause of compromised viability (S. Nottola, unpublished data).

In conclusion, in this article, we provided evidence that during slow cooling spindle and chromosome configurations are safely preserved provided that a higher sucrose concentration in the freezing solution is used. Our data also suggest that each individual protocol should be tested, considering that limited changes in the technical procedure may have significant consequences on the process of freezing and ultimately on oocyte quality. However, analysis on spindle and chromosome arrangement appears of relative value, being unable to provide full explanation to the clinical data that have been recently described. This calls for broader spectrum investigations on the quality of frozen oocytes.

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References


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