Compared to Vitrification, Slow Freezing Technique is Associated With a Higher Post-Thawed Embryos Survival and Clinical Pregnancy Rates. Is This a Myth or a Fact?

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**Abstract**

Objective: To compare the survival and clinical pregnancy rates of post-thawed human embryos, following cryopreservation using slow freezing and vitrification techniques.

Design: Retrospective cohort study.

Setting: Private In-Vitro Fertilization (IVF) centre, Saudi Arabia.

Patients: 516 patients who underwent IVF between June 2003 and June 2011 and had surplus embryos that were suitable for cryopreservation.

Method: Outcomes of interest were compared between embryos cryopreserved at cleavage stage (luteal day-3) by either slow freezing (322 patients) or vitrification (194 patients) techniques.

Results: The slow freezing group had significantly higher post-thaw embryo survival (69.75 vs. 62.75, *P*=0.012) and clinical pregnancy (34.8 vs. 21.5, *P*=0.002) rates.

Conclusions: In our experience, slow freezing of human embryos at cleavage stage (luteal day-3) yielded better results than vitrification. Larger controlled trials are needed to support this conclusion.

**INTRODUCTION**

Cryopreservation allows the transfer of limited number of fresh embryos back to the uterus while saving the remaining ones for future use; thus maximizing the cumulative effectiveness of an IVF cycle. Additionally, cryopreservation makes the postponement of embryo transfer (ET) to a future cycle feasible, thus decreasing the incidence of ovarian hyperstimulation syndrome (OHSS) in high-risk patients, while maintaining the probability of pregnancy [1].

The main problem during embryo cryopreservation...
is the formation of intracellular ice, which can lead to cell damage and developmental arrest. To overcome this problem, different cryopreservation protocols, such as slow freezing and vitrification, in addition to the use of different cryoprotectives, such as propanediol and dimethyl sulfoxide (DMSO), have been developed to protect cells against potential injuries occurring at subzero temperatures [2].

Currently, slow freezing is the most widely used method for cryopreserving human embryos. During this technique, embryos are exposed to combined controlled cooling rates with the use of low concentration of cryoprotectants. Alternatively, embryos can be cryopreserved by vitrification, a technique that combines ultra-rapid cooling with minimum volume along with use of high concentrations of cryoprotectants, allowing embryos to rapidly enter a glass-like state [3].

Since the publication of the first reports demonstrating the feasibility of using vitrification to cryopreserve human embryos, this method became increasingly popular among embryologists, because of its significant advantages with regards to cost and time requirements when compared to slow freezing [4]. The main concern remains however, is the need to use high concentrations of cryoprotective solutions that might lead to osmotic shock, which can affect embryo survival [5].

The feasibility of using vitrification to cryopreserve human embryos has far been shown by several groups, however the current best available evidence does not allow for solid conclusion to be made with regards to the association of this technique with higher pregnancy rate. Therefore, we aimed to review our data and assess the efficacy of slow freezing technique of human embryos and compare it to vitrification, in terms of post-thaw survival and clinical pregnancy rates.

MATERIALS AND METHODS

This is a retrospective cohort study conducted at a private In-Vitro Fertilization (IVF) centre in Saudi Arabia. Records from June 2003 until June 2011 were assessed and the data of 516 patients who underwent IVF and had surplus luteal day-3 (LD3) embryos that were suitable for cryopreservation was obtained. Institutional Ethical Committee approval was obtained.

Patients were prepared for oocyte retrieval using the standard ovarian long stimulation protocol. Down regulation was achieved using a gonadotropin releasing hormone (GnRH) agonist (Decapeptyl, Ferring, Germany), then ovarian stimulation was initiated using an exogenous recombinant gonadotropin (Puregon, MSD, Netherlands), with a starting dose of 225 to 300 International Unit (IU). The dose was then adjusted in tandem with ovarian follicular development as monitored by serial serum estradiol and transvaginal ultrasound (TV/US). Oocyte retrieval was performed 36 hours (h) after the administration of 10,000 iu of human chorionic gonadotropin (hCG, Chorimonon, IBSA, Switzerland), which was administrated when at least three follicles reached 17 millimetre (mm) in diameter.

After the oocytes were inseminated, resulting embryos underwent standard IVF protocols and were cultured in G1 (Vitrolife, Sweden), supplemented with 10% recombinant human serum albumin for 3 days. On LD3, embryos were scored according to a standard scoring system and labelled as Grade I, II, III or IV. Depending on the patient’s age, previous history, and embryo quality/grade, a maximum of 3 embryos were selected and cultured for 20 to 120 minutes (min) in Embryo Glue (Vitrolife, Sweden) prior to ET, which was later conducted by a standard ET catheter (Labotect, Straberg, Germany).

After ET, suitable remaining embryos were cryopreserved using slow freezing or vitrification techniques in 322 and 194 patients, respectively. During those years, all conditions and protocols for embryo culturing were kept constant.

In the slow freezing method, embryos were first incubated in an equilibration solution, comprising 1.5mol/L 1, 2 propanediol in Ham’s F-10 medium, supplemented with 20% Albuminal-5, containing 5% human serum albumin at room temperature for 10min, and then transferred to the freezing solution (1.5mol/L 1, 2 propanediol and 0.5mol/L sucrose in Ham’s F-10 medium), supplemented with 20% Albuminal-5 for an additional 10min. Thereafter, three or more embryos were loaded into a plastic mini-straw and the freezing program was executed. The straw was then held at -33°C for 30min before it was plunged and stored in liquid nitrogen (LN).

On the day of frozen ET (FET), one of the patient’s straws was removed from LN (before deciding to thaw the others), exposed to room temperature and then immersed in a water bath at 30°C. The embryos were then transferred from the straw to a decreasing concentrations of 1, 2 propanediol in the thawing solution (0.5mol/L sucrose and 20% Albuminal-5 in Ham’s F-10) for 5min and finally in sucrose-free thawing solution for another 5min before they were transferred to G1 culture media. FET was then conducted.

In vitrification, suitable surplus embryos were first incubated at room temperature in equilibration solution (7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO) in Ham’s F-10 media, supplemented with 20% Albuminal-5) for 5 to 15min (depending on the time needed for the re-expansion of cells). After an initial shrinkage and recovery, the embryos were transferred into vitrification solution (15% EG, 15% DMSO, 0.5 mol/L sucrose in Ham’s F-10) at room temperature for 60 seconds and embryos were immediately aspirated and placed at the tip of a Cryotop (Vitrolife, Sweden) and then plunged into LN. Three or more embryos were placed on each Cryotop.

On the day of FET, one of the patient’s Cryotops were rapidly removed from LN (before deciding to thaw the others) and the embryos were exposed to the thawing solution for 50 to 60sec at 37°C and then transferred (at room temperature) into dilution solution of 0.5 then 0.25mol/L sucrose for 3min each. The warmed embryos were washed 4-5 times into washing solution (Ham’s F-10 medium supplemented with 20% Albuminal-5) before they were transferred to G1 culture media. FET was then conducted.

Endometrial preparation was achieved using oral estradiol valerate (EV) starting with 4 milligram/day (mg/d) on menstrual days 1 to 5, then 6mg/d on MD 6 to 8 and finally with 8mg/d on MD 9 to 12. When the endometrial lining reached at least 8mm in thickness, micronized progesterone vaginal pessaries were
commenced at 100mg/d and EV was reduced to 4 g/d. Both were continued until 10 weeks of gestation or the commencement of a spontaneous menstrual period. FET was conducted three days after starting progesterone, and a pregnancy test followed by 15 days. If the latter was positive, a TV/US was performed 3 weeks later to confirm a clinical pregnancy by the presence of at least one intra-uterine gestational sac.

**STATISTICAL ANALYSIS**

Statistical analysis was made with the aid of Microsoft Excel. Differences amongst variables of the vitrification and slow freezing groups were analyzed using chi-square and Fisher’s exact tests for categorical variables, and the student’s t-test for continuous variables. P<0.05 was considered to be statistically significant. Comparisons of post-thawed survival and clinical pregnancy rates between the two groups were presented as odds ratios (OR) with corresponding 95% confidence intervals (95%CI). Levene’s test was used for assessing the equality of variances and t-test for equality of means.

**RESULTS**

Out of 516 patients, surplus embryos from 322 patients were cryopreserved using the slow freezing technique, while embryos from 194 patients were cryopreserved by vitrification. No significant difference in patients’ age was found between the two groups, however the mean storage duration was significantly longer and the mean number of frozen embryos was significantly higher for the slow freezing group (Table 1).

The post-thaw survival and clinical pregnancy rates were significantly higher for embryos cryopreserved using the slow freezing technique (69.75 vs. 62.75, P=0.012) and (34.8 vs. 21.5, P=0.002), respectively (Tables 2 and 3).

**DISCUSSION**

Cryopreservation of human embryos is a beneficial technique for assisted reproduction, as it provides a way to achieve a pregnancy, without exposing the female partner to ovarian stimulation or oocyte retrieval procedure, which are time consuming, stressful, costly, and are not without complications [6]. Currently, two cryopreservation methods are used, the “lengthy” slow freezing method that requires relatively expensive equipment, and the vitrification method that involves the use of highly toxic levels of cryoprotectants [7].

Although vitrification is becoming more attractive as it is relatively simpler and less expensive, slow freezing remains to be the most used technique for cryopreserving human embryos. Over the past few years, several studies with conflicting results were published comparing slow freezing with vitrification [8-11].

Our results showed that compared to vitrification, the slow freezing technique was associated with a significantly higher post-thaw survival and clinical pregnancy rates, although embryonic storage duration was significantly longer in this group. This may suggest that length of embryonic storage time have no detrimental effect on its survival or clinical pregnancy rates, which have also been suggested by Riggs et al [12]. Our group results also revealed that the number of cryopreserved 6 to 8 cells of grade I embryos after thawing were highly significant in the slow freezing group compared to the vitrification group with significant higher post-thaw survival and clinical pregnancy rates in the slow freezing method.

Randomized Controlled Trials (RCTs) comparing slow freezing versus vitrification techniques, in terms of post-thaw survival and clinical pregnancy rates had conflicting results (Table 4) [13-19]. Some agreed with our results, but others did not. All reviewed studies assessed the same primary outcomes measures we looked at. One study however, added live birth rate as a secondary outcome measure [14]. Those studies showed that vitrification appears to be better than slow freezing technique in terms of post-thaw survival rate, whether embryos were cryopreserved at blastocyst or cleavage stage, however the metaanalysis that included all those studies could not give solid conclusion with regards to the clinical pregnancy rate, and that is because two of the most relevant studies [14,19] that were part of this metanalysis, transferred embryos at different stages of development, which is a factor that can impact the clinical pregnancy rate, regardless of the technique used for freezing. Additionally, live birth rate, which was assessed by Rama et al, was not significantly different between the two freezing techniques [14]. Moreover, slow freezing technique was

### Table 1: Patients’ characteristics.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Slow Freezing</th>
<th>Vitrification</th>
<th>P-value</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>322</td>
<td>194</td>
<td>0.031</td>
<td>..........</td>
</tr>
<tr>
<td>Number of embryos produced</td>
<td>1607</td>
<td>750</td>
<td>0.043</td>
<td>..........</td>
</tr>
<tr>
<td>Age (years)</td>
<td>29.57±4.68</td>
<td>29.52±5.32</td>
<td>0.91</td>
<td>0.051</td>
</tr>
<tr>
<td>Duration of embryo storage in months (mean +/-SD)</td>
<td>11.08±1.08</td>
<td>6.79±4.72</td>
<td>0.001</td>
<td>4.3 (95%CI 2.68-5.91)</td>
</tr>
<tr>
<td>Number of frozen embryos (mean +/-SD)</td>
<td>7.47±3.48</td>
<td>5.53±2.38</td>
<td>0.001</td>
<td>1.94 (95%CI 1.43-2.45)</td>
</tr>
<tr>
<td>Blastocyst rate after thawing</td>
<td>0.00±0.00</td>
<td>0.24±0.82</td>
<td>0.001</td>
<td>0.237 (95%CI 0.34-0.12)</td>
</tr>
</tbody>
</table>

SD: standard deviation; OR: odds ratio; CI: confidence interval

### Table 2: Embryos’ survival after thawing.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Slow freezing</th>
<th>Vitrification</th>
<th>P-value</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>322</td>
<td>194</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survival rate (Mean ±SD)</td>
<td>69.75±28.06</td>
<td>62.75±31.89</td>
<td>0.012</td>
<td>7 (95% CI 1.55-12.45)</td>
</tr>
</tbody>
</table>

### Table 3: Clinical pregnancy rate.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Slow Freezing</th>
<th>Vitrification</th>
<th>P-value</th>
<th>*Clinical pregnancy rate (percentage)</th>
<th>34.8</th>
<th>21.5</th>
<th>0.002</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients who underwent FET</td>
<td>105</td>
<td>37</td>
<td></td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4: Summary of studies that compared Slow Freezing with Vitrification techniques.

<table>
<thead>
<tr>
<th>Study</th>
<th>Type of study</th>
<th>Outcome measures</th>
<th>Results</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li et al.13</td>
<td>RCT</td>
<td>PTS &amp; CPR</td>
<td>PTS: 88.8% CPR: 47.5%</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Rama et al.14</td>
<td>RCT</td>
<td>PTS, CPR &amp; live birth rate</td>
<td>PTS: 95.3% CPR: 35%</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Live birth rate:</td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>32.5%</td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Balaban et al.15</td>
<td>RCT</td>
<td>PTS &amp; blastulation rate</td>
<td>PTS: 94.9% Blastulation rate: 60.3%</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S</td>
</tr>
<tr>
<td>Zheng et al.16</td>
<td>RCT</td>
<td>PTS</td>
<td>PTS: 94%</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Huang et al.17</td>
<td>RCT</td>
<td>PTS &amp; CPR</td>
<td>PTS: 77.1% CPR: 53.8%</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Bernal et al.18</td>
<td>RCT</td>
<td>PTS &amp; CPR</td>
<td>PTS: 93% CPR: 62.5%</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Kuwayama et al.19</td>
<td>Prospective Cohort</td>
<td>CPR for cleavage stage embryos &amp; blastocysts</td>
<td>CPR: cleavage stage embryos 27% blastocyst 53%</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>study</td>
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</tbody>
</table>

RCT: randomised controlled trial; No.: number; Vit: vitrification; SF: slow freezing; PTS: post-thaw survival rate; CPR: clinical Pregnancy rate; NA: not available; S: statistically significant at P<0.05; NS: not significant

associated with a higher clinical pregnancy rate than vitrification in Kuwayama et al. study (32% vs. 27%), however the difference did not reach a statistically significant level [19]. Therefore, it cannot be said with confidence that vitrification is associated with a higher probability of clinical pregnancy [9].

In 2011 the UK Association of Clinical Embryologists (ACE) held a consensus workshop on Oocyte and Embryo Cryopreservation. The data supplied suggested that slow freezing and vitrification can lead to similar outcomes, regardless of the stage of embryos’ development (cleavage stage embryos or blastocysts), and the group concluded that at the moment there is not enough evidence to recommend either method [20]. The report however, stated that care should be taken when interpreting these data, because of the so many factors involved (other than the cryopreservation technique used) that could have influenced the results, such as the patient population differences, the method by which embryos for transfer were selected, and the number of embryos transferred. ACE added that well-designed trials with careful follow-up of born children are needed, before an optimal method can be agreed upon.

Different methods for vitrification have been described. Some of those methods ("open" methods) bring eggs and/or embryos into direct contact with LN in order to enhance the rate of cooling. It has been suggested that this may increase the risk of contamination with pathogens,[20] and that slow freezing may have a greater margin of safety [21]. Additionally, some clinics using the slow freezing technique showed data confirming that frozen cleavage stage embryos can survive with equivalent number of intact cells; as they were before crypreservation [21]. The same group reported high rates of survival of fully intact embryos, when the freezing and diluting solutions were modified, suggesting that FET can potentially be as effective as fresh ET, when it comes to clinical pregnancy rate [22]. Therefore, the current best available evidence assessing the relative efficacy of
vitrification versus slow freezing, does not support the shift from one technique to the other [23].

Another possible explanation for our findings is that our overall experience with slow freezing is significantly longer and greater than with the relatively “new” vitrification technique [8]. Centres may need to go through a learning curve before they get comfortable and achieve comparable results with vitrification. Additionally many vitrification protocols (as the one practiced in our centre), use high concentrations of permeating cryoprotectants such as DMSO. Usage of such cryoprotectants, variation in temperatures, as well as variation in the carrier system, may have a deleterious effect on the post-thaw embryo survival rates, which can potentially lower the clinical pregnancy rates as well.

CONCLUSION
Slow freezing may yield better results in experienced hands. Embryo cryopreservation is an area of active research, justifying the need for larger well-designed controlled trials to strengthen this conclusion, and to reliably evaluate the impact of each cryopreservation technique on the clinical pregnancy or even live birth rates, as well as on the potential impact on born children.

REFERENCES