Elective cryopreservation of all pronucleate embryos in women at risk of ovarian hyperstimulation syndrome: efficiency and safety

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In a prospective randomized study, we analysed 125 patients at risk of ovarian hyperstimulation syndrome (OHSS), selected in the period between January 1996 and July 1997. All the patients had blood oestradiol concentration \( \geq 1500 \text{ pg/ml} \) on the day of human chorionic gonadotrophin (HCG) administration and \( \geq 15 \) oocytes were collected. The patients were matched in two groups: group A, control group \((n = 67)\), had fresh embryo transfers; group B \((n = 58)\) had cryopreservation of all obtained pronucleate embryos. Pregnancy, live birth rates and the incidence of OHSS were compared between the two groups. There were no significant differences in terms of pregnancies per patient \((46.3 \text{ versus } 48.3\%)\) and live birth rates \((38.8 \text{ versus } 39.6\%)\). No cases of OHSS occurred in group B, while four patients developed the syndrome in group A. The implantation rate was slightly but not significantly lower in group B \((\chi^2 = 1.03)\). These results suggest that elective cryopreservation of all zygotes might prevent the risk of OHSS in patients undergoing IVF treatment. In contrast to what has been reported by other authors, our results show that the elective cryopreservation of zygotes does not affect pregnancy and live birth rates.

Key words: cryopreservation/embryo/ovarian hyperstimulation/thawing/zygotes

Introduction

Since the first case of ovarian hyperstimulation syndrome (OHSS) in in-vitro fertilization (IVF) patients, was reported (Friedman et al., 1984) it was clear that the syndrome was a complication of artificial induction of multiple follicular growth. It has also been widely established that the extensive use of gonadotrophin releasing hormone (GnRH) down-regulation protocols increases the risk of OHSS in infertile patients undergoing an IVF treatment cycle (Navot et al., 1992; Mordel and Schenker, 1993). Even though the incidence of the severe forms is low \((0.5-2\%)\), OHSS remains one of the major sources of morbidity and mortality in patients undergoing artificial reproductive technologies. Furthermore, other studies have clearly shown that the incidence and the duration of the syndrome increase in conceptional cycles as a consequence of prolonged ovarian exposure to endogenous human chorionic gonadotrophin (HCG) (Bassil et al., 1995; Gianaroli et al., 1996a). In order to reduce the risk of OHSS, several approaches have been proposed: reduction of ovulatory doses of HCG (Schenker, 1993); avoidance of luteal phase support with HCG (Araujo et al., 1995); administration of GnRH analogue during luteal phase (Gonen et al., 1990); prolonged coasting (Sher et al., 1993) or withholding gonadotrophin administration (Lee et al., 1998); early timed follicular aspiration (Tomazevic and Meden-Vrtovec, 1996); albumin administration at the time of egg retrieval (Asch et al., 1993); or, recently, i.v. hydroxyethyl starch solution at the time of embryo transfer (Koenig et al., 1998). Unfortunately, none of these methods has been demonstrated to have constant efficacy in preventing the syndrome except for the cancellation of the risky cycles before HCG administration. The latter, which cannot be easily determined by restricted criteria, is obviously frustrating for couples and a second attempt to induce multiple follicular growth could lead to the same risk. For these reasons, a new approach has been proposed, consisting of elective cryopreservation of all embryos and subsequent transfer of them in non-stimulated cycles (Amso et al., 1990). The controversial results reported by recent studies on this topic (Awonuga et al., 1996; Queenan et al., 1997) may reflect the different techniques and protocols used, or the different cut-off criteria applied to patients selected at risk of OHSS. Moreover, none of the published data have reported a prospective randomized trial comparing patients who had elective cryopreservation of all embryos versus those patients receiving fresh embryo transfers. In this study we present the results of a controlled randomized study performed to evaluate the safety and the efficacy of elective cryopreservation of all pronucleate embryos in patients at risk of OHSS compared to the fresh embryo transfer cycles in patients with similar potential risk of developing the syndrome.

Materials and methods

Between January 1996 and July 1997 all couples entering our centre for the first IVF attempt were thoroughly checked during the stimulation induction period in order to select those patients at risk of developing OHSS. All patients included in this study presented a high level of oestradiol the day of HCG administration \((\geq 1500 \text{ pg/ml or } \geq 5.500 \mu \text{mol/ml} \text{ (conversion factor to SI unit } = 3.671)\) and a high number of retrieved eggs \((\geq 15 \text{ oocytes})\). These parameters have been considered as a predictive cut-off for the surge of the OHSS based on a retrospective analysis of 4000 consecutive cycles performed in our centre. Out of 832 patients, 125 fulfilling the above-mentioned criteria were randomized into two groups: 67 patients \((\text{group A: control group})\) had fresh embryo transfers; 58 patients \((\text{group B: study group})\) had elective cryopreservation of all zygotes obtained.
The ovarian stimulation protocol was similar for all patients. We used a down-regulation protocol with GnRH analogue and urinary gonadotrophin (Metrodin; Serono, Rome, Italy) as described in our previous work (Ferraretti et al., 1996). All the patients were administered 7500 UI of HCG before egg retrieval. On the day of oocyte recovery, 20 g of i.v. albumin (Immuno A.G., Vienna, Austria) was administered in all cases as previously described (Asch et al., 1993). The oocytes were inseminated with the husband’s spermatozoa and then incubated in the same condition as reported previously (Gianaroli et al., 1996b). After 14–18 h from insemination the fertilization was assessed as shown by the presence of two pronuclei. All the obtained zygotes from group B patients were immediately cryopreserved, while the zygotes of group A couples were kept in culture medium for a subsequent 48 h. Oocyte culture, insemination and embryo culture were carried out in 100 µl droplets of IVF medium (Scandinavian IVF Science AB, Goeteborg, Sweden) overlaid with pre-equilibrated mineral oil (Scandinavian IVF Sc.). Three or four fresh embryos, according to the patient’s age, were transferred in group B, while the surplus viable embryos were cryopreserved. Pure progesterone in oil, 50 mg/day (Gestone; AMSA Laboratory, Florence, Italy), was given for luteal phase support from the day of embryo transfer.

All the studied patients were advised to rest at home and to monitor constantly the following parameters: weight, abdominal girth, fluid intake and urine output per 24 h. A controlled hyposodic and hyperproteic diet was also recommended. The patients were also asked to communicate the above parameters to the centre every 3–4 days or as soon as possible in cases of abnormal values or onset of symptoms such as nausea or vomiting.

Group A patients who failed the first fresh embryo replacement attempt and had the cryopreservation of surplus embryos, were submitted to a second attempt with thawed embryo transfer. These patients as well as the patients of group B, who had elective cryopreservation of all zygotes, underwent an appropriate hormonal replacement therapy (HRT) for the thawed cycle. The scheme included oral administration of oestradiol valerate (Progonova; Schering, Milan, Italy) 2 mg daily for the first 5 days of the cycle; 4 mg/day from day 6 to day 10; 6 mg/day from day 11 to day 13; then 4 mg/day from day 14 onward. On day 15 of the cycle, 50 mg of progesterone in oil was daily administered and on day 17 the dose was increased to 100 mg/day.

In group A patients, the cryopreserved embryos were thawed on day 17, and the viable embryos with ≥50% survived blastomeres were cultured in IVF medium for a few hours and then transferred immediately. Three or four cryopreserved pronucleate embryos of group B patients were thawed on the evening of the first day of progesterone administration and cultured for 36–40 h in IVF medium before embryo transfer. If two or more zygotes did not cleave 24 h after being cultured, one or two additional zygotes were thawed. The cryopreservation and thawing of either zygotes or embryos were performed using 1,2-propanediol and sucrose in phosphate-buffered saline solution (PBS) following the procedure previously described (Lassalle et al., 1985).

The patients who had frozen–thawed embryos transferred with a positive test of HCG continued the HRT for a further 8 weeks. The patients in whom fresh embryos were replaced and had a positive HCG test continued the progesterone administration until the first ultrasound examination. Only clinical pregnancies detected by the presence of gestational sac and fetal heartbeat, by ultrasound examination, were recorded and included in this study.

Oestradiol blood analysis was performed using a radioimmunoassay procedure (Biochem Immunosystem, Rome, Italy).

For statistical analysis, independent Student’s t-test and χ²-test were used where appropriate.

### Table I. Demographic data of group A and group B patients

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
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<tbody>
<tr>
<td>No. of patients</td>
<td>67</td>
<td>58</td>
</tr>
<tr>
<td>Age (years)</td>
<td>31.4 ± 2.4</td>
<td>31.6 ± 2.8</td>
</tr>
<tr>
<td>Duration of infertility</td>
<td>4.1 ± 2.9</td>
<td>3.9 ± 2.2</td>
</tr>
<tr>
<td>Male factor (%)</td>
<td>33 (49.2)</td>
<td>28 (48.3)</td>
</tr>
<tr>
<td>Tubal (%)</td>
<td>30 (44.8)</td>
<td>27 (46.5)</td>
</tr>
<tr>
<td>Others</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Parity</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PCO patients (%)</td>
<td>7 (10)</td>
<td>8 (14)</td>
</tr>
</tbody>
</table>

*a*Mean ± SD.

PCO = polycystic ovaries.

### Results

A total of 125 patients considered at risk of OHSS were selected and matched in two groups: 67 patients selected for transfer of fresh embryos (group A: control group) and 58 patients for elective cryopreservation of all obtained zygotes (group B: study group). There were no significant differences in the demographic data between the two groups (Table I). The stimulation parameters were also similar in terms of number of FSH ampoules administered, oestradiol peak at HCG administration, mean number of collected oocytes and fertilization rate (Table II).

In group A, 220 fresh embryos were transferred into 67 patients (mean ± SD: 3.2 ± 1.0 embryos/patient). In 13 cycles, 57 surplus viable embryos were cryopreserved (4.4 ± 2 embryos/frozen cycle). Clinical pregnancies were established in 28 patients (28/67 = 41.8%), with an implantation rate of 19%. Three pregnancies aborted during the first trimester and two resulted in ectopic implantation. Four patients in their conceptional cycles developed severe OHSS and required hospitalization. Eight couples of group A who failed the first attempt had a second frozen–thawed embryo replacement attempt. A total of 35 frozen embryos was thawed from which 24 viable embryos were obtained and replaced into the uterus of seven patients, producing three more pregnancies. Twenty-six pregnancies went successfully to term with the delivery of 39 healthy babies. The remaining 22 frozen embryos in group A belong to patients who have delivered.

In group B, a total of 609 fertilized eggs from 58 patients were cryopreserved at the pronuclear stage (10.3 ± 5 zygotes/patient). No severe OHSS developed in any of the 58 patients.
group B (χ^2 implantation rates were slightly lower, but not significantly, in the two groups in each step. The clinical pregnancy and the final clinical outcome of the zygotes and embryo performance achieved in both groups. The transfer of the surplus frozen–
of oocytes and embryos obtained from the two groups. Table III shows the outcome of spare cryopreserved zygotes. Table III shows the outcome of the first thawed cycle (20 out of 28), leading to a high number of healthy babies. Most of the pregnancies were achieved during while the remaining pregnancies resulted in the delivery of 30 (35.4% per cycle: 48% per patient) with an implantation rate of 185 embryos are still cryopreserved (22 in group A and 163 in group B). Because all these embryos belong to patients who have delivered, the study has to be considered completed and not affected by further attempts of these couples to obtain subsequent pregnancies with the use of the remaining zygotes or embryos.

### Discussion
Since the beginning of our IVF programme in 1985, our attention was addressed to the conflicting problem of OHSS that affects some patients undergoing an artificial follicular growth. In order to overcome severe cases of OHSS we have proposed a therapeutic approach, consisting in the use of dopamine (Ferraretti et al., 1992). The crucial and important point, however, is the prevention of the development of such syndrome. In our 13 years experience, the incidence of the severe form of OHSS is estimated 0.6% (27 cases out of 4445 stimulated cycles). Several approaches were used as preventive measures in order to reduce the incidence of the OHSS: i.v. albumin administration on the day of egg recovery as previously described (Asch et al., 1993), and prolonged coasting as previously described (Sher et al., 1993) and cycle cancellation of patients at high risk (oestradiol ≥5000 pg/ml). However, in our centre, the application of these preventive measures demonstrated a reduction of the incidence of OHSS from 0.8 to 0.5% associated with an increase of the stress for the couples because of the cycle cancellation or because of the reduced pregnancy rate due to the use of coasting.

A retrospective analysis of our data revealed that the incidence of the severity and the duration of the syndrome was strictly related to the surge of the pregnancy. In cycles where no conception occurred the incidence of OHSS was 0.3% with a mean duration of 6.2 ± 1.7 days, whereas in pregnant cycles 3.1% had OHSS lasting 16.4 ± 3 days. Furthermore, in pregnant patients, the incidence of OHSS is directly related to the number of implanted embryos: 1.8% in singleton; 2.5% in twins; 21% in triplet and 33% in quadruplet (Gianaroli et al., 1996a). Recent studies have proposed the elective cryopreservation of all generated embryos from patients considered at risk of developing OHSS, but the results achieved were contradictory. Some investigators suggested that this procedure reduces the incidence of severe OHSS and does not influence significantly the pregnancy rate (Pattinson et al. 1994). Other researchers argued that the elective cryopreservation of all embryos does not potentially prevent the development of OHSS, but reduces the pregnancy and live birth rates (Awnonga et al., 1996). In an additional study, (Queenan et al., 1997) it has been reported that despite the excellent pregnancy rate achieved with frozen–thawed embryos, the onset of OHSS is not avoided. These controversies could be explained with the different freezing procedures adopted, the different criteria used to define which patient is at risk and which is not, and the lack of a prospective randomized study. These findings encouraged us to perform a prospective randomized study to evaluate the efficiency as well as the safety of the elective freezing of all embryos in the prevention of the development of OHSS in stimulated patients. Data regarding the survival rate of embryos thawed at the zygote or cleaving stage, highlight the difference in terms of implantation capability, which is superior in the case of pronucleate stage embryos.

### Table III. Oocyte and embryo performance in group A and group B patients

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oocytes inseminated</td>
<td>1011</td>
<td>983</td>
</tr>
<tr>
<td>Oocytes fertilized</td>
<td>688 (68)</td>
<td>609 (62)</td>
</tr>
<tr>
<td>Cryopreserved 2PN</td>
<td>–</td>
<td>609</td>
</tr>
<tr>
<td>Fresh embryos cleaved</td>
<td>503</td>
<td>–</td>
</tr>
<tr>
<td>Fresh embryos transferred</td>
<td>220</td>
<td>–</td>
</tr>
<tr>
<td>Fresh embryos implanted</td>
<td>42 (19)</td>
<td>–</td>
</tr>
<tr>
<td>Embryos cryopreserved at 4–8 cells</td>
<td>57</td>
<td>–</td>
</tr>
<tr>
<td>Embryos thawed</td>
<td>35</td>
<td>446</td>
</tr>
<tr>
<td>Thawed embryos survived</td>
<td>28 (80)</td>
<td>351 (78)</td>
</tr>
<tr>
<td>Cleaving embryos after thawing</td>
<td>–</td>
<td>301</td>
</tr>
<tr>
<td>Thawed embryos transferred</td>
<td>24</td>
<td>240</td>
</tr>
<tr>
<td>Thawed embryos implanted</td>
<td>5 (21)</td>
<td>36 (15)</td>
</tr>
<tr>
<td>Spare embryos cryopreserved</td>
<td>22</td>
<td>163</td>
</tr>
</tbody>
</table>

Data are numbers with percentages in parentheses. PN = pronuclei.

### Table IV. Clinical outcome

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>67</td>
<td>58</td>
</tr>
<tr>
<td>No. of transfer cycles</td>
<td>74</td>
<td>79</td>
</tr>
<tr>
<td>No. of embryo per transfer cycle</td>
<td>3.2 ± 1.0</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>Clinical pregnancies</td>
<td>31</td>
<td>28</td>
</tr>
<tr>
<td>Pregnancy rate per patient (%)</td>
<td>46.3</td>
<td>48.3</td>
</tr>
<tr>
<td>Pregnancy rate per transfer cycle (%)</td>
<td>41.3</td>
<td>35.4</td>
</tr>
<tr>
<td>Implantation rate (%)</td>
<td>19.3</td>
<td>15</td>
</tr>
<tr>
<td>Live birth rate per patient (%)</td>
<td>38.8</td>
<td>39.6</td>
</tr>
</tbody>
</table>

*Mean ± SD.*
(Demoulin et al., 1991; Horne et al., 1997). Based on these observations, we decided to favour the cryopreservation of the zygotes and the subsequent embryo culture after thawing, with the aim of maximizing the chances of pregnancy in the patients studied. The results of the present study suggest that cryopreservation of 2PN zygotes does not affect significantly the developing embryo rate and does not decrease the chance of pregnancy. By adopting low cut-off parameters for the risk of OHSS, the cryopreservation of all zygotes seems to reduce the incidence of OHSS. The discomfort for the group B patients perhaps is higher with respect to group A: they must undergo delayed embryo transfer and a slightly higher number of embryo replacements in order to achieve a pregnancy rate similar to group A. Additionally, they have to continue the HRT for 7–8 weeks in case of pregnancy. However, in our centre these patients did not have additional costs, and the different incidence of OHSS compared to the control group seems to justify all these inconveniences.

From the current results, we cannot confirm that the elective freezing of all zygotes could eliminate completely the risk of the syndrome, but it reduces the duration and the severity in the complete absence of pregnancy. If costs and benefits of this procedure are taken into consideration, they should include the number of patients undergoing increasing stress due to the policy of elective cryopreservation of all embryos. In our centre, the proportion of patients with the parameters for elective cryopreservation of all embryos is quite consistent (~15%). On the other hand we believe that, if this procedure avoids only one case of severe OHSS in young healthy women, it is a proper strategy to be adopted, especially when the possibility of taking home a baby is not decreased.

In conclusion, our prospective randomized study demonstrates that the elective cryopreservation of all zygotes in patients at risk of OHSS is safe, as it reduces the risk of developing the syndrome, and is efficient as it does not affect the live birth rate. According to this study, our ongoing strategy is to cryopreserve all the pre-embryos at the pronucleate stage in patients considered at risk of OHSS.

References
Queenan, J.T., Veek, L.L., Toner, J.P. et al. (1997) Cryopreservation of all prezygotes in patients at risk of severe hyperstimulation does not eliminate the syndrome, but the chances of pregnancy are excellent with subsequent frozen–thawed transfers. Hum. Reprod., 12, 1573–1576.