Article

Laser-assisted hatching of cryopreserved–thawed embryos by thinning one quarter of the zona

Claudia Petersen is a graduate of the Master's programme in Clinical Embryology through the University of Leeds, UK. Since July 1990 she has been working as a permanent embryologist in the Centre for Human Reproduction, Prof. Franco Jr, Ribeirao Preto, Brazil, with focus on in-vitro culture and micromanipulation techniques. Her current research interests have focused on gamete and embryo morphology, implantation and cryobiology.

Dr Claudia Guilhermino Petersen

Claudia Guilhermino Petersen[1,2,3], Ana Lucia Mauri[2], Ricardo Luiz Razera Baruffi[1], João Batista Alcântara Oliveira[1], Valeria Felipe[1], Fabiana Cagnoto Massaro[1], Jose Gonçalves Franco Jr[1]
[1]Centre for Human Reproduction Prof Franco Junior, Av. Prof.João Fiusa 689–CEP 14025–310, Ribeirão Preto; [2]Post-graduate Fellow, Department of Gynecology and Obstetrics, Faculty of Medicine of Botucatu, UNESP, Botucatu, SP-Brazil
[3]Correspondence: Tel: +55 16 39111100; Fax: +55 16 39111100; e-mail: petersenclaudia@crh.com.br

Abstract

Laser-assisted hatching is little documented in the literature regarding its efficacy in cryopreserved–thawed (CT) embryo transfer cycles. The aim of the present study was to evaluate in a randomized manner the efficacy of thinning one quarter of the zona pellucida of CT embryos to a depth of 50–80% of the original thickness, via laser treatment (the qLZT-AH procedure), in improving implantation and pregnancy rates. Two populations were studied: population I, patients who had all their supernumerary embryos cryopreserved, regardless of their morphology, and population II, patients at risk of ovarian hyperstimulation syndrome who had all their embryos cryopreserved. Artificial and natural protocols were used for the embryo transfers. A total of 350 laser-thinned CT embryos were compared with 352 intact zona embryos. No difference in implantation or pregnancy rate was found after using qLZT-AH in either population. These findings suggest that qLZT-AH should not be routinely performed in cryopreserved embryo programmes.

Keywords: *cryopreserved–thawed embryos, implantation, quarter laser zona thinning*

Introduction

Embryo cryopreservation has been a successful part of assisted reproductive technologies, but the results appear to indicate a reduction in pregnancy and implantation rates compared with fresh embryos. It has been suggested that immersion in cryoprotectants and temperature changes during the process of cryopreservation may affect the physicochemical characteristics of the zona pellucida (ZP) by hardening it, and consequently may impair the natural process of blastocyst hatching (Schalkolff *et al*., 1989; Caroll *et al*., 1990; Vicente *et al*., 1990; Ilmmesse *et al*., 2005; Zech *et al*., 2005). Assisted hatching (AH) has been used to promote the embryo's ability to undergo hatching following the cryopreservation procedures (Cohen *et al*., 1991).

Comparative studies applying mechanical and chemical AH methodologies to cryopreserved embryos after thawing have been reported to be effective (Tucker *et al*., 1991; Check *et al*., 1996; Tao and Tamis, 1997; Vanderzwalmen *et al*., 2003; Gabrielsen *et al*., 2004) or not (Edirishinghe *et al*., 1999) in improving pregnancy and implantation rates. In the last 6 years, use of the non-contact infrared diode laser has become more frequent compared with previous AH methodologies, since it allows rapid, controlled and safe microdissection of the ZP (Rink *et al*., 1996; Benjamin *et al*., 2003). Several centres are applying this methodology for AH to fresh embryos and are also using it for embryo biopsies (Veiga *et al*., 1997; Baruffi *et al*., 2000; Mantoudis *et al*., 2001; Nagy *et al*., 2002; Petersen *et al*., 2002, 2005). However, a few randomized studies using laser methodology for cryopreserved–thawed (CT) embryos have

failed to show improvement in the implantation rate (Primi *et al*. 2004; Ng *et al*. 2005). The aim of the present study was to evaluate in a randomized manner the efficacy of thinning one quarter of the zona pellucida of CT embryos to a depth of 50–80% of the original thickness, via laser treatment (the qLZT-AH procedure), in improving implantation and pregnancy rates.

Materials and methods

Patients

The study was conducted on cryopreserved–thawed (CT) embryos from patients who were admitted to an intracytoplasmic sperm injection (ICSI) programme. A prospective randomized study of 258 frozen embryo cycles was performed at the Prof Franco Junior Human Reproduction Centre. According to a protocol approved by the Institutional Ethics Committee, two patient populations were included in this study. Population I comprised patients who had all their surplus embryos frozen, independently of the embryo morphology (number of cycles = 220); Population II included patients with risk of ovarian hyperstimulation syndrome (OHSS) who had all of their embryos cryopreserved (number of cycles = 38).

For both populations, whether a patient would be included in the qLZT-AH group or in the control group (no qLZT-AH) was determined by first using a randomized table previously elaborated for the study and then a second randomization by drawing lots at the time the patients were receiving their embryos, i.e. at the time of the transfer cycles. All transfers were performed by a single physician. An identifying code number was assigned to each patient at the time of randomization, in order to maintain anonymity.

The primary outcome measure was the evaluation of implantation rates and was defined as the number of gestational sacs seen by transvaginal ultrasound examination divided by the total number of embryos transferred. Clinical pregnancy, abortion and deliveries were calculated for each subgroup in both patient groups (qLZT-AH and control). A pregnancy test was performed on day 14 after treatment and clinical pregnancy was determined based on the presence of a gestational sac and fetal heart beat by ultrasound scanning 4 weeks after transfer.

ICSI and embryo culture

All patients were submitted to the routine scheme of ovarian stimulation (Franco Jr *et al*., 2001). Oocyte retrieval was performed 36 h after human chorionic gonadotrophin (HCG) administration by transvaginal ultrasound-guided aspiration. ICSI was performed according to the method of Svalander *et al*. (1995). Oocytes were examined after 17–20 h to assess fertilization and those with two distinct pronuclei were considered to be normal zygotes. Twenty-five to 27 h after injection, on day 1 of culture, early cleavage was evaluated and 2-cell embryos were separated for transfer (Petersen *et al*., 2001). For population I, embryo transfers were carried out on day 2 or day 3, according to the number of 4-cell embryo stages with equal regular blastomeres (grade 1 embryo) available on day 2. Day 2 embryo transfers were performed when three or fewer grade 1 embryos were available on day 2. On the other hand, if more than three grade 1 embryos were available on day 2, embryo transfers were carried out on day 3 in order to enhance embryo selection. However, for population II, all fresh embryos were cryopreserved on day 2.

Freezing procedure

Embryo cryopreservation was performed using a freezing media kit (Irvine Scientific, USA), which contained the following solutions: phosphate buffered saline (PBS), 1.5 mol/l propanediol (PROH) and 1.5 mol/l PROH + 0.1 mol/l sucrose. The material was cooled in a programmable freezer (Cryologic CL-863, Australia) with a starting temperature of 24°C. Cooling rate was 2°C per minute until –6°C, when manual seeding was performed. The temperature was then reduced 0.3°C per minute until –35°C, followed by a free fall to –150°C, after which the straws were transferred to liquid nitrogen.

Thawing procedure

For the thawing process, an embryo thaw media kit (Irvine Scientific) was used. Embryos were thawed by removing the straw from storage, exposure to air for 30 s and immersion in a water bath at 30ºC for 40 s. The embryos were successively added to different PROH solutions (1.0 mol/l PROH/0.2 mol/l sucrose, 5 min; 0.5 mol/l PROH/0.2 mol/l sucrose, 5 min) followed by 0.2 mol/l sucrose for 10 min. Rehydration was completed by transferring the embryos to PBS medium for 5 min at room temperature and to PBS stabilized at 37°C in the presence of 5% CO_2 for a further 5 min (Mauri *et al*., 2001; Fong *et al*., 2004). The criterion for the evaluation of morphological survival was that the thawed embryos would have at least one intact blastomere and no signs of damage to the zona pellucida (Lai *et al*., 1996). Surviving embryos were transferred to 50 μl microdroplets of preimplantation stage one medium (P1)/10% human serum albumin (HSA) or blastocyst medium/10% HSA (all Irvine Scientific, USA) in Falcon dishes, depending on the day they were frozen, and were placed in culture for 24 h at 37ºC/5.5% CO_2. Frozen–thawed embryo transfer was performed after assessment of embryo cleavage, when the division of at least one of the blastomeres was observed after 24 h of culture (Van der Elst *et al*., 1997). Embryos that presented a continuation of the embryo cleavage process were preferentially transferred. However, when such embryos were not available, embryos with no continuation of cleavage were also transferred. On the other hand, excess cleavage embryos were left in culture and refrozen at the blastocyst stage when available.

Embryo quality was assessed at the time of transfer for all patients according to the following criteria:

Grade 1: embryos with 8 cells by day 3 or the morula stage by day 4 without fragmentation and with all their blastomeres intact after thawing. Grade 2: embryos without 8 cells by day 3 and/or no morula stage by day 4 with ≥25% of their blastomere intact after thawing and/or with <25% fragmentation. Grade 3: embryos without 8 cells by day 3 or no morula stage by day 4 with >25% of their blastomere intact after thawing and/or >25% fragmentation.

Transfer cycles

Two different protocols were used for transfer.

In the natural cycle, follicular development was monitored by serial vaginal ultrasonography starting on day 10 of the cycle. A 10,000 IU dose of HCG was administered when a follicle of ≥17 mm was observed. Thawing was routinely performed on day 5 or 6 after HCG (day of HCG injection = day 0) and embryos were transferred on day 6 or 7 after HCG, depending on the day on which they were frozen.

In the artificial cycle, oestradiol valerate (Postoval; Wyeth, São Paulo, SP, Brazil) was administered from the first day to day 14 of the cycle at a daily dose of 6 mg. Progesterone (Utrogestan; Besins International, France) was also introduced on day 14 at a dose of 400 mg/day by the vaginal route, as long as endometrial thickness was ≥6 mm (Oliveira *et al*., 1997) and was increased to a daily dose of 800 mg on the day of embryo transfer. Thawing was performed on day 5 or 6 of progesterone treatment and embryos were transferred on day 6 or 7, depending on the day on which they were frozen.

Assisted hatching procedure

The embryos were positioned for the assessment of ZP thickness before laser manipulation and transfer. ZP thickness measurements were performed at four points (9, 12, 3 and 6 o'clock positions), using an inverted Eclipse TE 300 microscope (Nikon Instrument, NY, USA) equipped with a Hoffman lens and an ocular micrometer. qLZT-AH was performed with a 1.48 µm wavelength (infrared) diode laser (Fertilase™system; Medical Technologies Montreux, Lausanne, Switzerland) with a pilot laser light that operated through a ×40 microscope objective mounted on an inverted Eclipse TE 300 microscope with displacement heated stage. The embryos were treated directly in their original dish containing 50 µl microdroplets of culture medium, a Falcon 1006 Petri dish (Becton Dickinson, Denmark). qLZT-AH was performed by thinning the ZP to a depth of 50–80% of its thickness, starting at one point and continuing until 25% of the ZP was irradiated, i.e. laser thinning was initiated at the 9 o'clock position and consecutive irradiations were generated until the 12 o'clock position, with an irradiation time of 9 ms to reach a total length of approximately 80 µm.

Data analysis

Data are reported as means ± SD and were analysed using the InStat 3.0 program for Macintosh (GraphPad Software, San Diego, CA, USA). The Mann–Whitney test and Fisher's exact test were used when appropriate. The level of significance was set at $P < 0.05$.

Results

A total of 350 laser-thinned CT embryos were compared with 352 intact CT embryos. The patient characteristics such as age at embryo freezing, age at embryo thawing, infertility duration, primary and secondary infertility, aetiology, number of cycles, number of frozen embryos, number of previous transfers did not differ between the qLZT-AH and control groups or between populations I and II (**Tables 1** and **2** respectively).

Embryo factors such as survival and cleavage rates of CT embryos, mean number of embryos transferred and zona pellucida thickness were the same for the qLZT-AH and control groups and for populations I and II (**Tables 3** and **4** respectively).

For population I, a total of 20 clinical pregnancies (18.2%) and an implantation rate of 8.1% were achieved with the use of AH. This did not differ from the control group in which the thawed embryos were transferred with an intact zona (18.2 and 8.6%, respectively) (**Table 3**).

However, for population II, more patients achieved pregnancy when qLZT-AH was applied, although the rate was not statistically different from the control group (47.4 and 31.6% respectively). A higher implantation rate was also observed for laser-thinned embryos compared with controls, but without statistical significance (27.8 and 14.3% respectively) (**Table 4**). Deliveries and abortions were similar for the qLZT-AH and control groups and for both populations.

Embryo quality after thawing was evaluated in both populations. **Table 5** shows the percentage of grade 1, grade 2 and grade 3 embryos in population I and population II respectively. No significant difference in embryo quality was observed between the qLZT-AH and control groups in either population.

A detailed classification of grade 2 embryos was made in population I. No difference in the percentage of 2-cell embryos without fragments, 3–6 cells without fragments and 7 cells, 8 cells (day 4) or 9 cells without fragments embryos between qLZT-AH and control groups was observed (1.7 versus 3.0%; 47 versus 40%; 15.2 versus 18.5% respectively). For population II, the number of patients was too small for the presentation of similar data.

The implantation rate according to the embryo quality was evaluated in both populations. The implantation rate in patients who received only grade 1 embryos; at least one grade 1 embryo and no grade 1 embryos were 28.0 (7/25), 8.3 (17/204) and 7.0% (26/370) respectively in population I and 33.3 (3/9), 35 (13/37) and 10.5% (6/57) respectively for population II.

Two different schemes of transfer (natural and artificial) were used for both the qLZT-AH and control groups and both populations (I and II). In population I of the control group, more pregnancies were achieved when the embryos were transferred in an artificial cycle (27.1%) compared with natural cycles (7.8%) ($P = 0.01$, **Table 3**). However, this significant difference was not observed in the qLZT-AH group. In population II, there was no difference in implantation and pregnancy rates between the laser and control subgroup either for artificial or natural transfer cycles (**Table 4**).

Discussion

One of the reasons that has been suggested to explain the generally lower implantation rates after transfer of CT embryos compared with fresh embryos is the alterations that occur in the

Table 1. Characteristics of population I (all supernumerary embryos cryopreserved): comparison between the laser-thinned assisted hatching (qLZT-AH) group and the control group. There were no statistically significant differences between the two groups.

Characteristic	*qLZT-AH*	*Control*
No. patients	110	110
Patient age at embryo freezing (yrs)	31.7 ± 4.8	32.5 ± 4.4
Patient age at embryo thawing (yrs)	32.3 ± 4.0	33.4 ± 4.6
Infertility duration (yrs)	3.9 ± 2.7	4.7 ± 3.4
Primary infertility (%)	52	57
Secondary infertility (%)	48	43
Aetiology %		
Male	29	38
Female	44	38
Mixed	19	17
Idiopathic	8	7
No. previous cycles (%)		
0	10	11
1	44	41
2	20	22
≥3	26	26
No. previous cycles	2.2 ± 1.7	2.1 ± 1.5
No. embryos frozen	6.7 ± 3.9	6.5 ± 3.9

Values and means ± SD unless otherwise stated.

Table 2. Characteristics of population II (all embryos cryopreserved due to risk of ovarian hyperstimulation syndrome): comparison between the laser-thinned assisted hatching (qLZT-AH) group and the control group. There were no statistically significant differences between the two groups.

Characteristic	*qLZT-AH*	*Control*
No. patients	19	19
Patient age at embryo freezing (yrs)	32.1 ± 3.9	30.1 ± 4.3
Patient age at embryo thawing (yrs)	32.5 ± 4.0	30.5 ± 4.4
Infertility duration (yrs)	4.1 ± 2.5	4.8 ± 2.9
Primary infertility (%)	47	74
Secondary infertility (%)	53	26
Aetiology %		
Male	26	26
Female	42	53
Mixed	16	5
Idiopathic	16	16
Number of previous cycles (%)		
0	47	74
1	32	16
2	16	5
≥3	5	5
No. previous cycles	1.5 ± 0.7	1.6 ± 0.8
No. embryos frozen	15.9 ± 6.1	15.0 ± 6.0

Values and means ± SD unless otherwise stated.

Table 3. Outcomes of population I (all supernumerary embryos cryopreserved): comparison between the laser-thinned assisted hatching (qLZT-AH) group and the control group. There were no significant differences between the two groups. ZP = zona pellucida.

	qLZT-AH	*Control*
No. thawed transfer cycles	110	110
No. embryos thawed	448	457
Survival rate after thawing (%)	87	88
Cleavage rate after 24 h culture (%)	64	68
No. embryos transferred (mean ± SD)	2.69 ± 0.8	2.75 ± 0.7
Scheme of transfer (%)		
Natural	45	55
Artificial	55	45
ZP thickness (μm) of embryos transferred (mean ± SD)	17.1 ± 3.0	17.2 ± 2.7
Clinical pregnancy/transfer (%)	20/110 (18.2)	20/110 (18.2)
Clinical pregnancy/scheme of transfer (%)		
Natural	9/48 (18.8)	4/51 (7.8)[a]
Artificial	11/62 (17.7)	16/59 (27.1)[a]
Implantation rate/transfer (%)	24/296 (8.1)	26/303 (8.6)
Implantation rate/scheme of transfer (%)		
Natural	13/165 (7.9)	4/140 (2.9)[b]
Artificial	11/131 (8.4)	22/163 (13.5)[b]
No. abortions	4	4
No. deliveries	16	16

[a]$P = 0.01$; [b]$P = 0.003$.

Table 4. Outcomes of population II (all embryos cryopreserved due to risk of ovarian hyperstimulation syndrome): comparison between the laser-thinned assisted hatching (qLZT-AH) group and the control group. There were no significant differences between the two groups. ZP = zona pellucida.

	qLZT-AH	*Control*
No. thawed transfer cycles	19	19
No. embryos thawed	97	86
Survival rate after thawing (%)	91	87
Cleavage rate after 24 h culture (%)	70	70
No. embryos transferred (mean ± SD)	2.9 ± 0.6	2.6 ± 0.7
Scheme of transfer (%)		
Natural	32	21
Artificial	68	79
ZP thickness (μm) of embryos transferred (mean ± SD)	16.8 ± 3.1	17.2 ± 2.6
Clinical pregnancy/transfer (%)	9/19 (47.4)	6/19 (31.6)
Clinical pregnancy/scheme of transfer (%)		
Natural	2/6 (33.3)	2/4 (50.0)
Artificial	7/13 (53.8)	4/15 (26.7)
Implantation rate (%)	15/54 (27.8)	7/49 (14.3)
Implantation rate/scheme of transfer (%)		
Natural	3/18 (16.7)	2/11(18.2)
Artificial	12/36 (33.3)	5/38 (13.2)
No. abortions	2	1
No. deliveries	7	5

Table 5. Quality of embryos transferred. Population I (all supernumerary embryos cryopreserved); Population II (all embryos cryopreserved due to risk of ovarian hyperstimulation syndrome); qLZT-AH = laser-thinned assisted hatching. There were no statistically significant differences between the two groups within each population.

Embryos transferred	*Population I*		*Population II*	
	qLZT-AH	*No qLZT-AH*	*qLZT-AH*	*No qLZT-AH*
No. grade 1 (%)	47 (15.9)	62 (20.5)	13 (24.1)	13 (26.5)
No. grade 2 (%)	189 (63.9)	186 (61.3)	35 (64.8)	3 (65.3)
No. grade 3 (%)	60 (20.3)	55 (18.2)	6 (11.1)	4 (8.2)

glycoprotein matrix (zona pellucida) during the freezing–thawing process, causing the embryo to have 'exacerbated abnormal zona hardness', which results in failure of embryonic zona pellucida rupture, a prerequisite for implantation (Cohen *et al.*, 1991; Tucker *et al.*, 1991). AH has been used as a method for improving the implantation potential of these CT embryos with a 'harder zona', since it may improve the chances of the embryo attaching to the uterus by allowing earlier contact and dialogue between the embryo and the endometrium.

Conflicting information regarding the impact of AH on CT embryos exists in the literature. AH in CT human IVF embryos was first performed mechanically by Tucker *et al.* (1991), who reported a trend towards an improved implantation rate with the use of the partial ZP dissection methodology. Other groups later showed that chemical AH using acidic Tyrode's solution is an effective method for the improvement of implantation and pregnancy rates in thawed embryo transfers (Check *et al.*, 1996; Tao and Tamis, 1997; Gabrielsen *et al.*, 2004).

In contrast to the chemical AH method, for which only beneficial effects have been demonstrated, the effect of mechanical hatching on CT embryos has been reported to be beneficial (Vanderzwalmen *et al.*, 2003) or not (Edirisinghe *et al.*, 1999).

On the other hand, AH laser methodology has been successfully applied for the last decade in fresh embryo transfers (Antinori *et al.*, 1996; Petersen *et al.*, 2005; Ghobara *et al.* 2006). It permits a controlled and rapid microdissection of the ZP and its safety has been proved in both animals (Germond *et al.*, 1995) and humans (Germond *et al.*, 2000; Chatzimeletiou *et al.*, 2005). Laser irradiation produces photolytic ablation of the ZP without alteration of the cytoplasmic structure. Although it has been applied to many fresh embryos, its use for CT embryos has been described only in the last 2 years in two reports (Primi *et al.*, 2004; Ng *et al.*, 2005).

Primi *et al.* (2004), in a multicentre study, were the first to perform AH with a diode laser in CT embryos. The study was carried out at four IVF centres in which AH was performed on 405 embryos by opening their ZP in a single breach in patients coming for the first or third transfer cycle of CT embryos. For patients undergoing their first CT embryo transfer cycle, significantly lower pregnancy (1.6 versus 15.1%; $P = 0.01$) and implantation (1.6 versus 10%; $P = 0.01$) rates were obtained when they received laser-drilled embryos and no immunosuppressive/antibiotic treatment compared with control. In addition, patients coming for the third transfer cycle did not benefit from the use of laser methodology

Recently, Ng *et al.* (2005), in a randomized study using the same methodology as used in the present study, did not show improvement in implantation or pregnancy rates after transferring 188 laser-thinned CT embryos (9.0 and 12.5% respectively) compared with control (6.8 and 15% respectively), suggesting that laser AH should not be performed routinely in all CT embryos.

The data obtained in this prospective and randomized study failed to show a significant improvement in the implantation rate of CT embryos after using the qLZT-AH methodology in patients who had surplus or all embryos cryopreserved.

The present study agrees with the two previous studies using laser methodology. However, both previous studies showed an important bias that could affect the interpretation of the outcomes. In the study of Primi *et al.* (2004), patients submitted to pseudo-randomization based on historical control were used as control for the group of patients who were coming for the third transfer.

Furthermore, in the report of Ng *et al.* (2005), the randomization was not efficient in producing two groups (AH and control) with similar numbers of embryos transferred, suggesting that the clinical criteria defining the number of embryos to be transferred were not the same for the AH and control groups. Thus, both the historical control group (Primi *et al.*, 2004) and the imbalance in embryo number (Ng *et al.*, 2005) could affect interpretation of the outcomes.

On the other hand, the evaluation of the embryo development after the use of thinning assisted hatching methodology has been reported in the literature. Tinney *et al.* (2005), evaluating the development of 133 mouse embryos after applying laser zona thinning methodology (seven consecutive small holes in the ZP), did not show an increase in the hatching rate but rather resulted in a higher percentage of incompletely hatched embryos, suggesting that two small holes would prevent the complete hatching.

The quality of the embryo is a factor that can affect the outcome. An important point that should be noted when evaluating the outcomes is that in Brazil it is not permitted to discard poor quality embryos and all of the surplus embryos must be frozen regardless of their morphology. When a selected subpopulation of population I was evaluated, i.e. patients who received good quality embryos (≥2 cleaved embryos with ≥6 cell and >50% of the blastomeres intact), the implantation rate was apparently higher compared with the subpopulation who received poor quality embryos (≤1 cleaved embryo with <6 cells and ≤50% of the blastomeres intact) (11.6 versus 6.7%), but the difference was not statistically significant. The difference does suggest, however, that freezing of all surplus embryos may have impaired the results.

The data showed no significant difference in the quality distribution of CT embryos (grades 1, 2 and 3) in the qLZT-AH and control subgroups for either population I or II. Patients of population I received 15.9 and 20.5% of grade 1 CT embryos in the qLZ-AH and control groups respectively, and patients of population II received 24.1 and 26.5% of grade I CT embryos in the qLZT-AH and control groups respectively.

On the other hand, pregnancy and implantation rates were statistically different between populations I and II. Patients who had all their embryos frozen showed significantly higher pregnancy rates (39.5 versus 18.2%, $P = 0.005$) and implantation rates (21.3 versus 8.3%, $P = 0.0001$) compared with patients who had only surplus embryos cryopreserved. One interpretation of this fact could be the large number of oocytes collected from this population and the embryo quality. A higher number of grade 3 embryos (poor embryos) were transferred for the population with surplus embryos cryopreserved compared with this population (19.2% versus 9.8%, $P = 0.01$). There were no significant differences between the numbers of grade 1 and grade 2 embryos transferred in the two populations.

CT embryo transfers have been successfully performed in natural cycles following spontaneous ovulation and in cycles in which the endometrium is artificially prepared with exogenous steroids. Studies in the literature have compared the outcome of natural CT embryo transfers with artificial cycles, with conflicting results. Some groups have shown no difference in pregnancy rates using artificial cycles or not (Sathanantam *et al*., 1991; Al-Shawaf *et al*., 1993; Gelbaya *et al*. 2006), while others have shown higher clinical pregnancy rates in women who underwent artificial CT embryo transfer cycles (Schmidt *et al*., 1989; Muasher *et al*., 1991; Davies *et al*., 1991).

In the present study, no difference in outcome between artificial and natural cycles was observed for the qLZT-AH group (population I and II) and for control group of population II. However, a significantly higher pregnancy and implantation rate was obtained in artificial transfer cycles compared with natural transfers (27.1% and 13.5% versus 7.8% versus 2.9%, respectively, $P < 0.05$) for control group of population I. The difference can be explained by the wide variability in embryo quality between the groups. Patients whose transfers were performed in artificial cycles received a significantly higher percentage of good quality embryos (grade 1) compared with those whose transfers were performed in natural cycles (24.5 versus 13.6%, $P = 0.02$ respectively). On the other hand, patients whose transfers were performed in natural cycles received a higher percentage of poor quality embryos (grade 3) compared with artificial cycles transfers (23.5 versus 12.9%, $P = 0.01$). Grade 2 embryos were not different between natural and artificial transfers.

In the literature, a few randomized studies evaluating the efficacy of qLZT-AH on CT embryos with a large number of data are available. There is only one study assessing a total of 188 CT embryos. The data show the results of 350 microdissected embryos and can be helpful to evaluate the real efficacy of qLZT-AH for CT embryos.

In conclusion, qLZT-AH was not efficient regarding CT embryo cleavage either for patients who had supernumerary embryos or for those with all their embryos cryopreserved, and should not be routinely used in an IVF programme for cryopreserved embryos. However, its methodology has been an effective strategy for improving implantation of fresh embryos in patients with repeated implantation failures (Petersen *et al*., 2005).

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