Chapter 19
The Vitrification Component: An Integral Part of a Successful Single-Embryo Transfer Program

Juergen Liebermann

Introduction

In 1996, the Centers for Disease Control and Prevention (CDC) initiated data collection on assisted reproductive technology (ART). In 2011, the CDC reported a national multiple pregnancy rate with ART or in vitro fertilization (IVF) of 30 %, with 27.5 % rate for twin pregnancies and 2.5 % for triplet or higher-order multiples [1]. The original goal of IVF treatment was to maximize the chance of achieving a pregnancy by transferring several embryos, regardless of any known complications created by multiple-order pregnancies [1, 2]. It has since been shown that the risk of multiple-order births increases with the number of embryos being transferred, thus adversely modifying the risk for pregnancy complications [1, 3].

The increased risk of multiple pregnancies is therefore associated with increases in maternal and perinatal mortality and morbidity and increased costs for all parties involved [4–6]. Over the past decade, ART has made progress worldwide in terms of greater infertility treatment success [1, 3]. This can be attributed to the availability of complex culture media, a better understanding of in vitro culture conditions for human embryos, which allows culture to be maintained until the blastocyst stage, and improved cryopreservation techniques for surplus embryos not chosen for transfer [3]. But clinical experience shows that many patients are confronted with dilemmas when deciding whether to choose one or two embryos for embryo transfer. The nature of some of these dilemmas may lie in:

- The emotional stress that a patient may be undergoing (urgency to get pregnant)
- The financial aspects of the treatment (cost to the infertile couple, which increases with no IVF insurance coverage)

J. Liebermann (✉)
Fertility Center of Illinois, 900 N Kingsbury RW 6, Chicago, IL 60610, USA
e-mail: juergen.liebermann@integramed.com

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- Educational issues (lack of information about the risks of multiple gestations)
- Statistical concerns (being aware of the low ongoing pregnancy rate per treatment cycle from national data).

The European Society of Human Reproduction and Embryology Consensus Conference [7] raised awareness of the problem of infertility therapy-associated multiple pregnancies, suggesting that the essential aim of IVF "is the birth of a single healthy child, with twin pregnancy regarded as a complication" [7]. Accordingly, in an effort to reduce high-order multiple pregnancies, a growing body of evidence supports reducing the number of embryos transferred and moving toward elective single-embryo transfer (eSET) as a viable alternative to multiple-embryo transfers [8–15]. This may be thought of as moving away from simply "maximizing" an IVF cycle to "optimizing" an IVF cycle by maintaining a balance between the end result and the efforts, costs, and complications of the treatment. Besides carefully selecting the right patient, identifying the features that characterize a top-quality embryo is also crucial for achieving success with eSET [16, 17]. Today, the current established method for embryo selection in clinical application based on static morphologic and physical characteristics identified by light microscopy gets support by a variety of minimally invasive approaches such as time-lapse photography to assess "true" embryonic developmental potential. The application of time-lapse embryo monitoring under clinical application avoids the need to remove embryos from incubation to assess the embryo development on a daily basis. Moreover, by collecting time-lapse images and rewinding them in order to observe morphokinetic details in embryo development, an additional powerful tool for embryo selection exists.

Methods

Decision Making

What patient population would be suitable to offer eSET? The facts show that women with the best chance of getting pregnant after infertility treatment are also those at highest risk to conceive multiple gestations (usually patients who are age <35 years). The CDC revealed in their 2011 report that if patients younger than age 35 undergo a two-embryo transfer, the incidence of twin pregnancies was about 45 % with a occurrence of 1.3 % triplets or more [18]. At our institution, we recommended using eSET for good prognosis patients. The criteria for this recommendation include:

- Age <37 years
- Having their first IVF cycle or having conceived in a previous IVF cycle
- Availability of one or more high-quality blastocysts.

Furthermore, convincing patients to reduce the number of embryos transferred from two to one is effective only when a patient is convinced of the success of eSET. The acceptance of eSET can be supported by a successful cryopreservation program which can achieve outcomes similar to that of fresh transfers.

The Vitrification Procedure

The impact of cryopreservation on the growth and improved efficiency of assisted reproduction in humans has become increasingly appreciated. With approximately one-quarter of a million babies born following cryopreservation, cryopreservation has been shown to increase pregnancy rates while allowing for further selection of embryos. Therefore, it is possible to achieve implantation and pregnancy rates with frozen–thawed embryos as high as those achieved with fresh embryos. Lower numbers of embryos are being transferred, resulting in fewer higher-order multiple gestations and improved implantation rates. Moreover, cryopreservation of embryos is a powerful tool in the prevention of twins. In addition, the true augmentation potential of cryopreserving embryos on the total reproductive potential of a single oocyte harvest can be evaluated.

Today, cryopreservation is one of the keystones of clinical infertility treatment. In particular, an ultrafast cooling technique known as vitrification has become a well-established and widely used procedure that allows important expansion of therapeutic strategies during IVF. Most important, vitrification of human blastocysts allows the potential for conception to be maximized from any one in vitro fertilization cycle and prevents wastage of embryos. The ability to vitrify blastocysts either on day 5 or day 6 opens the opportunity to offer to selected patients the transfer of one elective single blastocyst instead of two, with no decrease in pregnancy rate while also greatly reducing the likelihood of multiple gestation. Making the patient aware, and the key here lies in the importance of patient education in regard to multiple pregnancies, of the nonexistence of different outcome using either fresh or vitrified blastocyst will increase their confidence in the procedure and in their choice to go with one embryo at a time.

Next, the application of vitrification technology for cryopreserving human blastocysts is described in a step-by-step sequence.

Stepwise Blastocyst Vitrification Procedure

Vitrification of blastocysts should be undertaken utilizing a “closed system” (HSV: High Security Vitrification Kit; CryoBio System, L’Aigle, France; FDA 510(k) clearance for cleavage stage embryos in blastocysts) after a two-step loading with cryoprotectant agents at 24 °C. If assisted collapsing is done before vitrification, then the blastocyst should be placed on an inverted microscope equipped with a

laser system (ZILOS-tk, Hamilton Thorne). The junction of two trophectoderm cells in each blastocyst needs to be located and one pulse (100 % power, 500 µs duration) applied. Then the blastocysts are returned to the incubator for 5–10 min. Briefly, blastocysts should be placed in equilibration solution, which is base medium (M199 with 20 % Serum Supplement Substitution, SSS) containing 7.5 % (v/v) ethylene glycol (EG) and 7.5 % (v/v) dimethyl sulfoxide (DMSO). After 5–7 min, the blastocysts need to be washed quickly in vitrification solution, which is the base medium containing 15 % (v/v) DMSO, 15 % (v/v) EG, and 0.5 M sucrose, for 45–60 s and transferred onto the HSV using a micropipette. Immediately after the loading of not more than two blastocysts in less than 1 µl drop on the HSV, the straws can be heat sealed, then plunged in LN_2, and secondarily stored inside 5 ml liquid nitrogen prefilled canes (Visotube Rond, IMV; France). Each component is described in detail below.

1. Aseptic techniques are required at all stages. For equilibration and vitrification procedures ensure the benchwarmer is at room temperature (~25 °C).
2. Take reagents from the refrigerator and allow them to warm to room temperature.
3. Separate the blastocysts to freeze into a separate well. Bring this dish to the inverted microscope and with the embryo positioned with the laser objective use a single pulse to hit the blastocysts between two trophectoderm cells to collapse the embryo. Place the dish back into the incubator for 5–10 min.
4. Label a petri dish with the patient's name under the lid as follows: HTF-HEPES, ES, and VS. Prepare 2 × 50 µl of HTF-HEPES, 2 × 50 µl of ES, and 4 × 50 µl of VS.
5. The vial label should include the patients' first and last name, accession number, MPI#, date plus number and type of embryos.
6. Before vitrification, use a Stripper tip with 200 µm end hole for loading the blastocysts on the top.
7. Fill Styrofoam container with LN_2.
8. Each sample that is vitrified will be done in a separate hood and verified by a second embryologist before proceeding. Vitrify good expanded/hatching blastocysts on day 5/6/7.
9. Remove embryos from culture dishes using a stripper tip into the HTF-HEPES (drop 1), gently aspirating to remove any traces of culture media.
10. Pipette from mHTF (drop 1) to the other drop of mHTF (drop 2) and immediately merge it with the first drop of ES (drop 3). Set timer for 5 min.
11. When the time is up, transfer embryos to the remaining drop of ES (drop 4). Set the timer for 3 min. Place embryos on the top of the drop and let them settle to the bottom.
12. Next, load blastocysts in a VS back-loaded stripper tip and rinse through the four droplets of VS (drops 5–8), between each droplet clean tip.
13. Note that placement into the VS and loading of the cryotop should take <1 min, so that the total incubation time in 15 % VS is 30 s. After 30 s, gently transfer them to the tip of the HSV by using a stripper tip to load the blastocysts in as small volume as possible (i.e., <0.5 µl) onto the edge of the stick.

14. Visually confirm placement.
15. Before loading, apply label to the open end of the empty straw. Load the HSV stick into the empty straw, the side with the embryos first. Use the blue handle to make sure the stick has been fully advanced. Then, using the heat sealer, seal the open end of the stick and plunge the whole straw into the LN_2. Place the straw in a precooled aluminum cane for further storage.
16. Store cane in nitrogen tank.
17. Record cane location on the freezing worksheet and cryo inventory log.
18. Complete all paperwork and recheck that all vial locations are logged into the Embryo Inventory.

Stepwise Blastocyst Warming Procedure

Regardless of the day of cryopreservation of the embryo (whether day 5, 6, or 7), at thawing, blastocysts should be treated as if they had been frozen on the fifth day of development. To remove the cryoprotectants, blastocysts need to be warmed and diluted in a three-step process. With the HSV submerged in LN_2, the inner straw should be removed. The carrier with the blastocysts can then be removed from the LN_2 and placed directly into a pre-warmed (37 °C) organ culture dish containing 1 ml of 1.0 M sucrose. Blastocysts can be picked up directly from the HSV and placed in a fresh drop of 1.0 M sucrose at 24 °C and immediately connected with a drop of 0.5 M sucrose. After 5 min, blastocysts can be transferred to 0.5 M sucrose solution and connected with drops of base medium for additional 5 min. Even when switching the cells between different concentrations of warming solutions, fill up the pipette with the next lower concentration of warming solution before picking up the cells for moving in the following concentration. Then the blastocysts can be washed in the base medium for 3 min and returned to the culture medium (SAGE Blastocyst Medium, Trumbull, CT, USA) until transfer. Each single step is described in detail below.

1. Take reagents from the refrigerator and allow them to warm to room temperature. All cryoprotectants are removed at 25 °C.
2. Place a 200 µl drop of TS on a petri dish and place on a warming plate.
3. Label a petri dish (Nunc) with the patient's name under the lid as follows: TS, DS, and WS. Prepare 1×50 µl of TS, 4×50 µl of DS, and 6×50 µl of WS.
4. Before warming, use a Stripper tip with 200 µm end hole for removing the blastocysts from the top.
5. Fill Styrofoam container with LN_2.
6. Confirm location and identification with a second embryologist before warming any HSV kit. Warm one kit at a time.
7. Each sample that is warmed will be done in a separate hood and verified by a second embryologist before proceeding.
8. With the HSV kit under LN_2, open the kit by cutting the outer straw. Use the blue handle to remove the inner stick.

9. Submerge HSV kit directly in the pre-warmed drop containing TS, which should be as close as possible to the LN_2 styrofoam container. As soon as the HSV kit contents liquefy (within 1 min), try to locate the blastocysts before removing them with a stripper tip. After locating all the blastocysts, remove them from the tip and place them in the droplet of TS (drop A) and connect immediately with the first droplet of DS (drop B). Wait for shrinkage and re-expansion.
10. When they start to wrinkle, connect with the second droplet (drop C) and finally with third droplet of DS (drop D).
11. When they stop reacting and start to reshrink, transfer blastocysts to 0.5 M sucrose (drop E) by placing at the top of this drop so they float to the bottom. When the reaction is complete, connect with first of WS (drop F; wait for about 90 % re-expansion).
12. After 100 % expansion, connect with droplet #2 (drop G) and then with droplet #3 (drop H) of WS. Turn on benchwarmer and finally dilute through a series of three wash drops of HS (I to K).
13. Place the blastocysts into a culture dish and put it in the incubator for subsequent culture.
14. Record the survival and appearance of all blastocysts. Update log with warm data, and notify the physician of result.

Results

Successful Application of eSET

Since 2007, Fertility Centers of Illinois (FCI) offered eSET using morphologic criteria for the selection of good-quality embryos, combined with careful selection of patients. The following report summarizes the results of our study with eSET at this institution.

Between 2007 and April of 2014, we performed 8,192 autologous cycles with embryo transfer of which 3,453 (42 %) embryo transfers were performed on day 3 without eSET, and 4,739 (58 %) embryo transfers were done on day 5. Records of a total of 1,037 autologous eSET on day 5 (~22 % of all blastocyst transfers—1,037:4,739) were reviewed. The CDC reported a national average of 12.2 % for eSET cycles in patients age <35 years in 2011 [19].

The mean (±SD) age of our patient population was 31.8 ± 3.3 years. On average, 18 oocytes per patient were retrieved. Of a total of 18,173 oocytes retrieved, 80.1 % were injected, and 77.0 % fertilized normally (11,207/14,551). The majority (98.0 %) of the fertilized oocytes cleaved on day 2. Of normally fertilized oocytes, 74.0 % progressed to blastocyst stage (Table 19.1). In 1,037 eSET cases, 717 positive pregnancies (69.1 %) were achieved with 642 clinical pregnancies (62.0 %). The implantation rate was 63.5 % (659/1,037), with 579 ongoing pregnancies, yielding a 55.8 % ongoing pregnancy rate. We have now confirmed live births from 883 eSETs done between 2007 and July of 2013. The ongoing pregnancy rate was 53.7 % (474/883), followed by a live birth rate of 51.8 % (457/883).

Table 19.1 Retrospective outcome data from 1,037 autologous elective single-embryo transfers on day 5

Patients, *N*	1,037
Patients' age, years	31.8 ± 3.3[a]
Oocytes retrieved, *N*	18,173
Oocytes injected, *N*	14,551
Oocytes fertilized, *N* (%)	11,207 (77.7 ± 14.6[a], 76.8–78.6[c])
Embryos cleaved on day 2, *N* (%)	10,967 (98.1 ± 5.9[a], 97.7–98.5[c])
Embryos with ≥6 blastomeres on day 3, *N* (%)[b]	9,714 (87.2 ± 15.4[a], 86.3–88.1[c])
Compacting embryos on day 4, *N* (%)[b]	8,219 (74.0 ± 22.0[a], 72.7–75.3[c])
Blastocysts on day 5, *N* (%)[b]	8,270 (74.3 ± 18.3[a], 73.2–75.4[c])
Patients who underwent eSET, *N*	1,037
Implantations, *N* (%)	659 (63.5)
Positive pregnancies/eSET, *N* (%)	717 (69.1)
Clinical pregnancies/eSET, *N* (%)	642 (62.0)
Ongoing pregnancies/eSET, *N* (%)	579 (55.8)
Multiple pregnancy rate, *N* (%)	17 (2.6)
Confirmed live births from 883 eSET between 2007 and July 2013	
Ongoing pregnancies/883 eSET, *N* (%)	474 (53.7)
Live birth rate/883 eSET, *N* (%)	457 (51.7)
Live births, *N*	469 (211 boys and 258 girls)

eSET elective single-embryo transfer, *SEM* standard error of mean
[a]Means ± SEM; [b]%/2 pns; [c]95 % confidence interval

In all of the 1,037 eSET, blastocysts were available for transfer on day 5. Of note, 963 patients had cryopreservation (93 %) whereas only 74 patients (7 %) ended up with no cryopreservation at all. It should be mentioned that 30 % of the "no cryopreservation" group had no cryopreservation because they declined to sign the relevant consent. A total of 4,961 blastocysts were vitrified, yielding an average of five blastocysts per patient.

Applying eSET to a large proportion of patients, more embryos would be available for vitrification which in turn would result in more successful vitrified–warmed cycles. The majority of blastocysts were vitrified on day 5 (67.5 %), whereas only 32.5 % were vitrified on day 6 (see Table 19.2). As shown in Table 19.2, patients with no embryos suitable for cryopreservation had the same chance to get pregnant compared with the group of patients having surplus embryos for cryopreservation.

Results on Blastocyst Vitrification After Failed Fresh eSET

To calculate a patient-specific augmented pregnancy rate, it is essential to include as augmentation only those pregnancies from vitrified blastocysts among patients who did not have a pregnancy after fresh eSET. This represents true augmentation of the patient-specific expectation of pregnancy from the same oocyte harvest. After 1,037 eSET, a total of 464 patients experienced a negative outcome. 320 patients

Table 19.2 Retrospective outcome data from 1,037 autologous elective single-embryo transfers on day 5 with or without having embryos suitable for cryopreservation

	1,037	
Total number of eSETs	With cryopreservation	Without cryopreservation
Patients, *N*	963	74
Patients' age, years	31.7 ± 3.2[a]	32.0 ± 4.3[a]
Blastocysts vitrified, *N*	4,961	–
Average number of blastocyst per patient vitrified	5	–
Day 5 blastocysts vitrified, *N* (%)	3,347 (67.5)	–
Day 6 blastocysts vitrified, *N* (%)	1,614 (32.5)	–
Implantations, *N* (%)	612 (63.6)*	47 (63.5)
Positive pregnancies/eSET, *N* (%)	667 (69.3)*	50 (67.6)
Clinical pregnancies/eSET, *N* (%)	597 (62.0)*	45 (60.8)
Ongoing pregnancies/eSET, *N* (%)	535 (55.6)*	38 (51.4)

*$p > 0.05$
[a]Means ± SEM

Table 19.3 Retrospective outcome data from 333 autologous vitrified–warmed embryo transfers after failed fresh elective single-embryo transfers compared with 1,037 fresh day 5 eSETs

	FET after failed fresh eSET	Fresh eSET
Patients, *N*	333	1,037
Patients' age, years	32.1 ± 3.1[a]	31.8 ± 3.3[a]
Blastocysts warmed, *N*	594	–
Patients taking ONE embryo, *N* (%)	76 (23)	–
Patients taking TWO embryos, *N* (%)	253 (76)	–
Patients taking THREE embryos, *N* (%)	4 (1)	–
Implantations, *N* (%)	278 (46.8)	
Patients with single implantation, *N* (%)	134 (65.0)	
Patients with twin implantation, *N* (%)	73 (35)**	17 (2.6)
Positive pregnancies/eSET, *N* (%)	234 (70.3)*	717 (69.1)
Clinical pregnancies/eSET, *N* (%)	207 (62.2)*	642 (62.0)
Ongoing pregnancies/eSET, *N* (%)	192 (57.7)*	579 (55.3)
Cumulative ongoing pregnancy rate/oocyte retrieval, *N* (%)	192 + 579 = 771/1,037 = 74.3 %	
Added value of cryopreserving embryos (%)	74.3 − 55.8 = 18.5	

FET frozen embryo transfer
*$p > 0.05$; **$p < 0.001$
[a]Means ± SEM

(30.1 %) faced a negative pregnancy test, 75 patients achieved a biochemical or ectopic pregnancy only, and additional 69 patients lost their ongoing pregnancy beyond 7 weeks. To reflect the true augmenting effect of vitrification, only the first transfer of vitrified embryos occurring after an unsuccessful fresh eSET was analyzed. 333 patients returned for a frozen embryo transfer; 70.3 % achieved a positive pregnancy, with a clinical and ongoing pregnancy rate of 62.2 % and 57.7 %, respectively (see Table 19.3).

Because more than 70 % of the 333 patients subsequently elected to undergo a two-embryo transfer, the occurrence of twins increased to 35 %. However, combining the ongoing pregnancy rate from the fresh eSET (579) and the first frozen transfers (192) provides the cumulative expectation of a pregnancy with embryos from the same oocyte retrieval of 74.3 % (771:1,037). As shown in Table 19.3, the added value of cryopreservation is 18.5 % (74.3–55.8). At FCI, extended culture generates high pregnancy and implantation rates, even when we are transferring just one embryo instead of two. After 1,037 eSETs at our center, we did not observe a decrease in the overall ongoing pregnancy rate in our program, although a dramatic reduction of twins was observed (from 48 % in 980 fresh transfers in patients age <35 years with two blastocysts versus to <3 % with fresh eSET) and a complete disappearance of any high-order multiple pregnancies (see Tables 19.1 and 19.3).

Discussion

These data show that successful implementation of eSETs for clinical application can be achieved. Patient education concerning the risk of multiple gestations is important, as is the acceptance of eSET among physicians and embryologists [19]. In many ways, the success of an IVF unit's eSET program is contingent on having suitable cryopreservation skills available in the laboratory. Taken together, attitude, acceptance, and equipment are therefore essential ingredients to implement eSET successfully and to effectively reduce the rate of multiple gestations associated with IVF. Implementation of ESET at our institution has been shown to be a valuable tool, not merely to maximize but rather to *optimize* pregnancy rates. Initiating a patient education program is a top priority to establish a successful eSET program.

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Conflict of Interest The author discloses no conflict of interest.

References

1. CDC Report. Assisted reproductive technology surveillance—United States. 2011. http://www.cdc.gov/art/ARTReports.htm.
2. Faber K. IVF in the US: multiple gestations, economic competition, and the necessity of excess. Hum Reprod. 1997;12:1614–6.
3. Adashi EY, Barri PN, Berkowitz R, et al. Infertility therapy-associated multiple pregnancies (births): an ongoing epidemic. Reprod Biomed Online. 2003;7:515–42.
4. Oleszczuk AK, Oleszczuk JJ, Keith LG. Defining the high-risk nature of triplet pregnancies. Int J Fertil Womens Med. 2002;47:182–90.

5. Ombelet W, Martens G, De Sutter P, et al. Perinatal outcome of 12,021 singleton and 3108 twin births after non-IVF-assisted reproduction: a cohort study. Hum Reprod. 2006;21:1025–32.
6. Doyle P. The outcome of multiple pregnancy. Hum Reprod. 1996;11 Suppl 4:110–7.
7. Land JA, Evers JL. Risks and complications in assisted reproduction techniques: report of an ESHRE consensus meeting. Hum Reprod. 2003;18:455–7.
8. Martikainen H, Tiitinen A, Tomás C, et al. One versus two embryo transfer after IVF and ICSI: a randomized study. Hum Reprod. 2001;16:1900–3.
9. Tiitinen A, Unkila-Kallio L, Halttunen M, Hyden-Granskog C. Impact of elective single embryo transfer on the twin pregnancy rate. Hum Reprod. 2003;18:1449–53.
10. Gerris J, De Neubourg D, Mangelschots K, et al. Elective single day 3 embryo transfer halves the twinning rate without decrease in the ongoing pregnancy rate of an IVF/ICSI programme. Hum Reprod. 2002;17:2626–31.
11. Gerris J, De Sutter P, De Neubourg D, et al. A real-life prospective health economic study of elective single embryo transfer versus two-embryo transfer in first IVF/ICSI cycles. Hum Reprod. 2004;19:917–23.
12. Van Landuyt L, Verheyen G, Tournaye H, et al. New Belgian embryo transfer policy leads to sharp decrease in multiple pregnancy rate. Reprod Biomed Online. 2006;13:765–71.
13. De Sutter P, Van der Elst J, Coetsier T, Dhont M. Single embryo transfer and multiple pregnancy rate reduction in IVF/ICSI: a 5-year appraisal. Reprod Biomed Online. 2003;6:464–9.
14. Criniti A, Thyer A, Chow G, et al. Elective single blastocyst transfer reduces twin rates without compromising pregnancy rates. Fertil Steril. 2005;84:1613–9.
15. Gerris J. Single-embryo transfers versus multiple-embryo transfer. Reprod Biomed Online. 2009;18:63–70.
16. Hunault CC, Eijkemans MJ, Pieters MH, et al. A prediction model for selecting patients undergoing in vitro fertilization for elective single embryo transfer. Fertil Steril. 2002;77:725–32.
17. Gardner DK, Surrey E, Minjarez D, et al. Single blastocyst transfer: a prospective randomized trial. Fertil Steril. 2004;81:551–5.
18. US Department of Health and Human Services and Centers for Disease Control and Prevention Assisted Reproductive Technology. Success rates national summary and fertility clinic reports. 2011. http://nccd.cdc.gov/DRH_ART/Apps/NationalSummaryReport.aspx.
19. Henman M, Catt JW, Wood T, et al. Elective transfer of single fresh blastocysts and later transfer of cryostored blastocysts reduces the twin pregnancy rate and can improve the in vitro fertilization live birth rate in younger women. Fertil Steril. 2005;84:1620–7.