Reproductive BioMedicine Online (2015) **30**, 378–384

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ARTICLE

Non-synchronized endometrium and its correction in non-ovulatory cryopreserved embryo transfer cycles

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Abstract The aim of this case series study was to investigate the effect of adjusting the length of progesterone exposure on clinical pregnancy rates in cryopreserved embryo transfer cycles of patients with out-of-phase classic endometrial dating. Eighty infertile women with previous implantation failure and good-quality embryos underwent endometrial biopsy before cryopreserved embryo transfer and were included in this study. The main outcome measures were clinical pregnancy rate and histologic endometrial dating. After adjusting the length of progesterone exposure according to endometrial dating, a significantly higher implantation rate was observed in blastocyst transfers ($P = 0.02$) and the clinical pregnancy rate for all cycles was 36.4%, similar to that in patients with in-phase endometrium (22.5%). In conclusion, the use of classic histologic endometrial dating to estimate the timing of the window of implantation and to adjust progesterone exposure accordingly may increase the implantation rate in frozen embryo transfer cycles.

KEYWORDS: cryopreserved embryo transfer, endometrial dating, pregnancy, window of implantation

http://dx.doi.org/10.1016/j.rbmo.2014.12.005
1472-6483/

Introduction

For a human pregnancy to occur, a normally developing embryo needs to implant successfully on the uterine endometrial surface. The endometrium must be in a receptive state for implantation to occur and to support further embryonic development. The endometrium acquires receptivity to embryo implantation in response to progesterone action on an appropriately oestrogen-primed endometrium. Oestrogenic stimulation results in endometrial proliferation and the induction of progesterone receptors (Lessey et al., 1988; Nastri et al., 2012). The exposure of the endometrium to progesterone starts a series of morphological and functional alterations that transform the lining from a proliferative to a secretory epithelium. The secretory glands elongate and increase the rate of secretion of products essential for implantation. In humans, the 'window of implantation', the time in which the endometrium is most conducive to trophoblast-endometrial interactions, is thought to be restricted to days 22–24 of an idealized 28-day cycle (Bergh and Navot, 1992). The morphological changes characteristic of each day after ovulation have been described by Noyes et al. (1975), and have established the basis for classic histologic endometrial dating that served in the past 6 decades as the gold standard for clinical evaluation of luteal function.

Implantation is the key step in initiating a successful pregnancy (Cakmak and Taylor, 2011; Simon et al., 1998). Many factors that were shown to affect the success of implantation after embryo transfer, including the quality and morphological grade of embryos (Karlstrom et al., 1997), a series of synchronized changes in the endometrium leading to endometrial receptivity, (Modi et al., 2012; Strowitzki et al., 2006) and the technique of embryo transfer (Angelini et al., 2006). It has been estimated that a large proportion of implantation failures occur because of defects in endometrial receptivity (Simon et al., 1998).

In natural cycles, the endometrial lining of the uterus starts preparing for embryo implantation in the proliferative phase and becomes synchronized with embryo development during the luteal phase (Strowitzki et al., 2006). Implantation occurs 8–10 days after ovulation (Wilcox et al., 1999).

Cryopreservation of human gametes and embryos has become an essential part of assisted reproduction since the first child was born after frozen embryo transfer in 1984 (Zeilmaker et al., 1984). Success with frozen embryo transfer cycles significantly increases the overall cumulative pregnancy rate per retrieval. Also cryopreservation of all embryos can be an effective solution for women at high risk of ovarian hyperstimulation syndrome (D'Angelo, 2010) or in need of fertility preservation such as patients diagnosed with cancer (Rossi et al., 2011).

Traditionally, frozen–thawed embryo transfers were associated with a lower pregnancy rate compared with fresh transfers because of less than optimal embryo survival after slow freezing for cryopreservation, and the fact that the highest quality embryos are generally selected for a fresh transfer (Mocanu et al., 2008). One other potential cause of failure of implantation in cryopreserved embryo transfers, however, is the artificial preparation of the endometrium, which, unlike fresh cycles, is dissociated from the development of the embryos.

In preparation for cryopreserved embryo transfers, it is customary to administer oestrogen until the thickness of the lining has reached an optimal level (about 0.8–1.4 cm), and then to add progesterone for the number of days proportional to the stage of development of the embryo/s being transferred (Paulson, 2011). This practice follows the assumption that all women after being primed with oestrogen and exposed to progesterone for the proper number of days will develop a synchronised endometrial lining. An endometrial biopsy that shows a difference of more than 2 days between the histological dating and actual day after ovulation is considered to be 'out of phase' (Wentz, 1980). In previous publications, out-of-phase endometrium was found in less than 5% to 50% of patients (Coutifaris et al., 2004; Sahmay et al., 1995; Zawar et al., 2003). These studies, however, report on endometrial biopsies carried out during a natural cycle, and at least some of the results that were interpreted as being 'out of phase' might be attributed to inaccurate determination of the time of ovulation. In endometrial preparation for cryopreserved embryo transfers, most patients receive high-dose oestrogen treatment administered during the follicular phase to inhibit gonadotrophin secretion, and to prevent follicular development and ovulation. Alternatively, a gonadotrophin-releasing hormone (GnRH) agonist is administered to suppress gonadotrophin secretion during endometrial preparation. Consequently, any shift in endometrial development can, for the most part, be accurately attributed to altered endometrial response to hormonal preparation.

The aim of the present study was to determine if clinical pregnancy rate could be increased in women with previous failed embryo transfers of good-quality embryos by using the classical histological dating of the endometrial lining in a mock (no embryo transfer), non-ovulatory, cycle that included endometrial preparation with oestradiol and progesterone. The results of the endometrial dating were used in a subsequent cycle to adjust the length of exposure to progesterone to compensate for apparently out-of-phase endometrial responses in the mock cycle.

Materials and methods

This case series was conducted at the Toronto Center for Advanced Reproductive Technology, a University of Toronto affiliated IVF clinic. The study was approved by the Mount Sinai Hospital institutional ethics review board on 30 November 2011 (reference: 11–0268-C).

Inclusion criteria were previous failed embryo transfer, including fresh and cryopreserved embryos with at least one good-quality embryo transferred, based on previously described criteria (Cutting et al., 2008). The number of previous embryos transferred were as recommended by the Society of Obstetricians and Gynaecologists of Canada and the Canadian Fertility and Andrology Society (SOGC-CFAS, JOINT, 2008). Previous failed cryopreserved embryo transfers were included based on the study by Shapiro et al. (2013), which described a higher pregnancy rate for the cryopreserved embryo transfers compared with fresh embryo transfers. Finally, at least one cryopreserved, good-quality embryo had to be available (Cutting et al., 2008). Exclusion Criteria were abnormal uterine cavity per sonohysterogram and presence of uni- or bilateral hydrosalpinx.

Embryos were cryopreserved either by slow freezing (Mazur, 1990) or vitrification (Vajta and Nagy, 2006). The Toronto Centre for Advanced Reproductive Technology has been using vitrification since 2012; however, some cryopreserved embryo transfers that were carried out during the study period included embryos that were slow frozen before 2012.

Women scheduled for a cryopreserved embryo transfer cycle, who had previously failed to conceive in a fresh cycle and in one or two prior cryopreserved embryo transfers, were asked to complete a mock transfer cycle with endometrial biopsy for endometrial dating in which the endometrium was prepared using oestrogen and progesterone. Informed written consent was obtained. The women received micronized oestradiol, starting from cycle day 2 (Estrace; Shire Canada Inc,Saint-Laurent, QC) 4 mg a day by mouth for 5 days, followed by 8 mg a day for at least 5 days until an endometrial thickness 8 mm or more was reached with a triple line pattern. The dose of oestrogen was high enough to prevent follicular development. Progesterone, in the form of vaginal suppositories (Kingsway Drugs, Toronto, Ontario, Canada) 200 mg three times daily was then started, and the oestradiol reduced back to 4 mg a day. After 6–7 days of the oestrogen and progesterone combination, an endometrial biopsy was carried out using a pipelle endometrial suction curette (Cooper Surgical-USA) and sent for histological examination.

The endometrial biopsy was followed by 7 more days of oestrogen and progesterone administration. After cessation and induction of a withdrawal bleed, the actual frozen embryo transfer treatment cycle began. This protocol was the same for all of the patients included in the study whether they had a synchronized endometrium or not.

In the frozen embryo transfer treatment cycle, patients started the identical oestrogen priming and vaginal ultrasound monitoring to measure endometrial thickness. By then, the results of the histological examination of the lining had been received. Patients with an in phase endometrium according to histological dating underwent FET after 4–6 days of progesterone and oestradiol depending on the developmental stage of the embryo to be transferred (day 3 or blastocyst, respectively).

Vaginal progesterone was continued for 2 more weeks until a pregnancy test. In women with out-of-phase endometrium, defined as a discrepancy of 2 days or more between the observed and expected endometrial dating, the number of days of progesterone administered before frozen embryo transfer was adjusted to compensate for either delayed or advanced endometrial dating. As the results of the dating biopsy were given as a range of 1–2 days, it was left to the treating physician's discretion to choose the final added number of days of progesterone administration.

Embryo transfer was carried out using double lumen soft catheter (COOK catheter) (COOK Inc, ON, Canada) and was ultrasound guided. The number of embryos transferred was according to accepted guidelines (2008). After the transfer, patients continued on progesterone and oestradiol until the pregnancy test 2 weeks later. If the pregnancy test was positive, progesterone suppositories 200 mg twice a day were continued until 10 weeks' gestation. Some women, who failed to conceive after the first mock cycle and subsequent cryopreserved embryo transfer cycle, underwent a second mock cycle with endometrial biopsy followed by another cryopreserved embryo transfer cycle, as recommended by their physician.

Assessment and outcome measures

Baseline clinical data of patients were analysed, including age, parity, days of correction needed, number and quality of embryos transferred and pregnancy outcome. The endometrial samples were examined by external histopathologists who were blinded to the information on the length of progesterone exposure. The histopathologist was asked to date the endometrial samples. The analysis was based on the daily sequential changes outlined by Noyes et al., 1975) in their classic study. Dating the endometrium involves identifying morphological changes characteristic for early, middle, and late proliferative phase endometrium and for each of the 14 days of secretory endometrium. Delays or advancements were identified in relation to the actual days of progesterone administration in the mock cycles. When the delay or advancement was for more than 2 days, the endometrium was considered to be out of phase.

Histological endometrial dating and the clinical pregnancy rate were considered as primary outcome measures. In all patients who became pregnant, the number of gestational sacs and viability of the pregnancy was confirmed using transvaginal ultrasound. Clinical pregnancy was confirmed by the presence of fetal cardiac activity on transvaginal ultrasonography at 6–8 weeks' gestational age. The implantation rate was calculated by dividing the number of living fetuses on ultrasonography by the number of embryos transferred. Clinical spontaneous abortion was considered when loss of pregnancy occurred before 20 weeks of gestation (Regan and Rai, 2000). Data were analysed using Microsoft Excel version 7. Data were described in terms of mean and SD; $P < 0.05$ was considered statistically significant.

Results

In this study, a total of 84 patients underwent 101 endometrial biopsies in mock cycles before frozen embryo transfer cycles. Basic characteristics of the patients and the cryopreserved embryo transfer data are detailed in **Table 1**. A total 59 patients was shown to have in-phase endometrium in 74 biopsies. Another 21 patients were shown to have delayed endometrium in 23 biopsies. Another four patients with four biopsies showing endometrial advancement were not included in the data analysis, owing to their small number and because of an error on our part in adjusting the progesterone.

Reproducibility of the histological dating was confirmed, as 13 patients underwent one additional biopsy and two patients underwent two additional biopsies. All women had the same results of either in-phase or delayed endometrium on the repeat biopsies as seen with the first biopsy.

The outcomes for both groups of patients are shown in **Table 2**. For the 59 patients with in-phase endometrium, 71 frozen embryo transfer cycles were carried out, in which no correction of the length of progesterone administration was needed. Of the 71 transfers, 16 patients conceived (clinical pregnancy rate per transfer [22.5%]). Multiple gestational sacs in one patient were counted as one clinical pregnancy.

Table 1 Patient characteristics and cryopreserved embryo transfer data for in-phase and delayed endometrium groups. No statistically significant differences were found between the two groups.

	In-phase (n = 59)	*Delayed endometrium (n = 21)*
Age (years) mean ± SD	37.2 ± 5.0	37.3 ± 4.2
Number of patients with previous pregnancy (%)	36/59 (61)	15/21 (71)
Number of biopsies	74	23
Number of previous failed embryo transfers, including fresh and frozen mean ± SD	2.3 ± 1.7	2.5 ± 1.8
Number of previous failed fresh embryo transfer mean ± SD	1.2 ± 0.7	1.2 ± 0.4
Number of cryopreserved embryo transfer cycles	71	22
Number of embryos transferred mean ± SD	2.4 ± 0.8	1.8 ± 0.8
Number of top quality embryos transferred (number of transfers) mean ± SD		
Day 6	4 (3)	
Day 5	43 (22) 2.1 ± 0.6	16 (8) 2.0 ± 0.5
Day 3	97 (46) 2.0 ± 0.6	28 (14) 2 ± 0.8

Table 2 Outcomes in patients with in-phase endometrium and delayed endometrium with progesterone adjustment.

	In-phase	*Delayed endometrium*
Clinical pregnancy rate per transfer *n* (%)	16/71 (22.5)	8/22 (36.4)
Implantation rate *n* (%)		
Day 3	17/97 (17.5)	3/28 (10.7)
Day 5	6/43 (13.9)	7/16 (43.8)[a]
Spontaneous abortion rate per clinical pregnancy *n* (%)		
Day 3	8/13 (57.1)	1/3 (33.3)
Day 5	1/3 (33.3)	2/5 (40.0)
Live births	seven deliveries; eight babies (one twin delivery); 7 /71 (9.9%)	2/22 (9.1%) in addition to one termination because of a congenital heart defect, one still birth and one second- trimester spontaneous abortion.

[a]$P = 0.02$.

Table 3 Clinical pregnancy rate and days of correction in delayed endometrium corrected cycles. Multiple gestational sacs in one patient are counted as one clinical pregnancy.

Endometrial delay (days)	*Number of patients (number of embryo transfers)*	*Days of progesterone administration before embryo transfer*		*Clinical pregnancy Rate per transfer (%)*
		Day 3 embryo	*Day 5 embryo*	
−2	6 (6)	6 days		
−3	10 (10)	4–7 days	9 days	3/10 (30.0%)[a]
−4/−5	5 (6)	6–8 days	9 days	5/6 (83.3%)[b]

[a]Includes one twin pregnancy, day 5 embryo transfer after 7 days of progesterone.
[b]Includes one twin pregnancy, 4 days delayed endometrium, day 5 embryo transfer after 9 days of progesterone.

In the 21 patients with delayed endometrium (23 biopsies: −5/−4 days delayed in six biopsies, −3 days in 11 biopsies, −2 days in six biopsies), progesterone exposure was adjusted according to the endometrial biopsy results. Of 22 FETs, eight patients conceived (**Table 2**), giving a clinical pregnancy rate per transfer of 36.4%.

The clinical pregnancy rates according to the length of endometrial delay are shown in **Table 3**.

No pregnancy was achieved in patients with −2 days delay, three conceived out of 10 transfers in patients with −3 days delay (30.0%), and the highest pregnancy rate was observed in the −4/−5 days delay group (83.3%).

Delivery outcomes are shown in **Table 2.** Seven successful deliveries took place, with eight live born babies out of 71 cryopreserved embryo transfer cycles (9.9%) in the in phase group. In the delayed endometrium group after progesterone adjustment, two successful deliveries took place with two live born babies out of 22 cryopreserved embryo transfer cycles (9.1%). No significant difference occurred between two groups.

Discussion

The criteria for endometrial dating were defined by Noyes et al. in 1950, and have since become the gold standard for endometrial dating (Noyes et al., 1975). Over the years, multiple studies have criticised the accuracy and reproducibility of this method and questioned its role as part of the basic evaluation of female infertility (Coutifaris et al., 2004; Murray et al., 2004). Previous studies addressing endometrial dating were carried out on cycling patients, with the reference point of ovulation defined either retrospectively by assuming that ovulation occurred 14 days before the menstrual period, or by detection of the LH surge by ovulation prediction kits or basal body temperature. Since none of these methods is completely accurate, some of the variability detected in endometrial dating likely stems from misdiagnosis of ovulation. In this study, endometrial dating was carried out on endometrium artificially prepared by sequential exposure to oestrogen and progesterone on non-cycling patients. Exactly the same protocol was followed for all the patients included in the study. Incorrect detection of ovulation and the stage of the cycle could therefore be eliminated as a source of bias.

The endometrial samples were examined and dated by external histopathologists who were blinded to the information on the length of progesterone exposure. The analysis was based on the Noyes' criteria (1975) that are the gold standard for endometrial dating and have been for many years.

In this study, it was shown that, despite the artificial preparation of the endometrium for implantation, 23 out of 97 (23.7%) of the endometrial samples were diagnosed as delayed endometrium by 2 or more days (up to 5 days in some cycles). Another four samples that were diagnosed as advanced endometrium but, owing to an error on our part in adjusting the progesterone, these were not included in the analysis.

The number of out-of-phase endometria is similar to previous reports (Coutifaris et al., 2004; Keenan et al., 1989). Because of the non-ovulatory nature of the preparation of the lining, it can concluded that it is not a dysfunctional corpus luteum or a lack of progesterone that are the cause of non-synchronization but rather an internal defect of the individual endometrium. The same pattern of the endometrium (i.e. being in phase or out-of-phase in repeated biopsies in the same patients) showed a 100% concordance in the present study. Adjusting the number of days of pre-transfer progesterone administration according to the dating of the endometrial biopsy resulted in a clinical pregnancy rate similar to the in-phase endometrium group.

With current embryology techniques, top quality, euploid embryos may be transferred with no implantation taking place. This suggests that more attention be directed towards understanding the non-receptive endometrium (Casper, 2011).

Both oestrogen and progesterone are necessary to prepare the endometrium for implantation (Paulson, 2011). The initiation of pregnancy requires synchrony between endometrial development and the implanting blastocyst (Simon et al., 2000). Experience acquired with frozen–thawed as well as donor embryo transfer, in which no functional corpus luteum exists, showed that only the sequential administration of oestrogen and progesterone is needed to achieve successful implantation (Shapiro et al., 1993). Oestrogen administration in the first part of the cycle allows for endometrial proliferation and suppression of folliculogenesis. Progesterone administered in the second part of the cycle suppresses proliferation and induces cell differentiation (Bergeron, 2000; Wang et al., 1998).

Previous studies attempted to establish a relationship between endometrial thickness, endometrial pattern (Jarvela et al., 2005) and pregnancy rate in IVF cycles, as evaluation of the endometrium by ultrasound is both simple and non-invasive (Bergh et al., 1992; Gonen and Casper, 1990). In the present study, progesterone was added after endometrial preparation with oestrogen and only after its thickness had reached at least 8 mm with a triple-line pattern (Oborna et al., 2004). Endometrial biopsy was carried out 6–7 days after starting progesterone. A previous study showed that the timing of the biopsy in the luteal phase does not affect its accuracy (Coutifaris et al., 2004).

In the present study, one-quarter of endometrial samples were found to be advanced or delayed in spite of the oestrogen preparation of the endometrium, and accounting for the days of exposure to progesterone. Correction of endometrial synchronization by adjustment of the length of progesterone exposure before frozen embryo transfer led to a significantly higher implantation rate in blastocyst transfers ($P = 0.02$) and a similar clinical pregnancy rate of 36.4% compared with cycles with in phase endometrial biopsies. This outcome was comparable to the overall previous frozen embryo transfer cycle (2007–2010) pregnancy rate of 24.3%.

A recent study by Nawroth and Ludwig (2005) highlighted the significance of accurately timing the progesterone exposure, with both early and late progesterone exposure having detrimental effects on the outcome of donor embryo as well as frozen thawed embryo transfers. One could argue that the endometrial biopsy itself may have increased the chance for pregnancy, as suggested by Nastri et al. (2012). A beneficial effect on implantation would have applied to both in-phase and out-of-phase biopsy groups.

Multiple studies have attempted to identify an accurate method of assessing endometrial receptivity to to increase live birth rates in assisted reproduction (Casper, 2011). The endometrium has a unique quality of actively preventing embryo implantation outside the period of time defined as the window of implantation. The transition between out-of-phase, non-receptive to in-phase, receptive endometrium is complex and requires multiple changes (Achache and Revel, 2006). Previous studies showing a large variability in endometrial histological dating and lack of association with infertility have led researchers to seek other markers of endometrial receptivity (Achache and Revel, 2006; Coutifaris et al., 2004; Garrido-Gomez et al., 2012). As discussed above, however, some of this variability may stem from a misdiagnosis of the timing of ovulation. Endometrial biopsy can also be important in excluding endometrial pathology that may interfere with embryo implantation (Johnston-MacAnanny et al., 2010). For example, in the present study, a patient with in-phase endometrium was diagnosed with endometritis and achieved pregnancy after treatment with a course of antibiotics.

One of the limitations of this study was the lack patients with out-of-phase endometrium with no correction of the length of progesterone treatment. The decision not to include such a group was based on two assumptions. The first was that all of the patients included in the study had previously failed a cryopreserved embryo transfer before the mock cycle, where

the length of progesterone exposure was not corrected. Second, after the biopsy result was received, not correcting the progesterone treatment would have meant that the embryos for these patients would have been knowingly transferred outside the presumed window of implantation, and we were uncomfortable with this possibility.

In conclusion, it was found that, endometrial non-synchronization, determined by simple histologic endomentrial dating and the ability to correct it by simply adjusting the duration of progesterone exposure, led to an improved implantation and pregnancy rate in frozen embryo transfer cycles. This observation suggests a possible role for endometrial dating (by the standard Noyes criteria), which is widely available in all pathology laboratories, as a simple tool in fertility assessment, at least in the case of hormonal preparation of the endometrium for cryopreserved or donor oocyte cycles, where adjustment of the day of embryo transfer is possible and could improve the outcome of cryopreserved embryo transfers.

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Declaration: The authors report no financial or commercial conflicts of interest.

Received 15 June 2014; refereed 2 December 2014; accepted 4 December 2014.