A simple, inexpensive and effective artificial cycle with exogenous transdermal oestradiol and vaginal progesterone for the transfer of cryopreserved pronucleated human oocytes in women with normal cycles

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Supernumerary pronucleated stage oocytes (PN) are usually cryopreserved. PN are transferred in spontaneous, stimulated or artificial cycles. In this study, an artificial cycle with a transdermal therapeutic system was used for oestradiol release (Estraderm TTS 100®) in combination with a targeted drug delivery system for vaginal progesterone release (Crinone 8%®). Patients started transdermal 17β-oestradiol treatment on cycle day 1. Only one clinical monitoring was necessary on day 14 for confirmation of satisfactory endometrial development and exclusion of ovulation by transvaginal ultrasound and endocrine determinations (oestradiol, progesterone and luteinizing hormone). Embryo transfer was performed on the third day of progesterone treatment (day 17). The first 25 cycles were recently completed in a prospective study; no cycles were cancelled due to ovulation or unsatisfactory endometrial development. In comparison with the previous protocol of embryo transfer in stimulated cycles in our clinic which required extensive ultrasound and endocrine monitoring, the pregnancy rate in these oestrogen- and progesterone-supplemented cycles was nearly twice as high (34.8%). Two pregnancies were even achieved with zygotes after microinjection of frozen–thawed late spermatids extracted from testicular tissue (cryo-TESE). In these cycles, the Estraderm TTS 100/Crinone 8% protocol seems to be superior to stimulation protocols and even to other protocols reported so far for artificial cycles with exogenous oestradiol and progesterone treatment.

Key words: artificial cycle/cryopreserved pronucleated human oocytes/targeted drug delivery/transdermal therapeutic system/vaginal progesterone gel
Introduction

To avoid multiple pregnancies, the German Medical Association recommends that for patients aged <35 years no more than two embryos should be transferred. Moreover, according to the embryo protection law in Germany, the number of embryos transferred in in-vitro fertilization (IVF) cycles is limited to three. Thus supernumerary pronucleated stage oocytes (PN) are usually cryopreserved. However, cryopreservation of embryos at the cleavage stage (which offers the chance of embryo selection) is also prohibited.

Pregnancy rates after embryo transfer of cultured frozen–thawed cryopreserved zygotes are unacceptably low in spontaneous or stimulated cycles. Moreover, daily ultrasound and hormone determinations are necessary to monitor ovulation. Embryo transfer must be carried out 2 days after ovulation, so that it cannot be planned in advance. Thus, we were searching for an alternative protocol that should be simple, convenient for the patient, inexpensive and, above all, successful.

PN oocytes are usually thawed, cultured for up to 24 h and transferred at the 2- or 4-cell stage. In spontaneous or stimulated cycles, the embryo transfer is carried out 2 days after ovulation (Dale and Elder, 1997). Successful controlled preparation of the endometrium in artificial cycles can be achieved either with ‘modulated’ oestrogen replacement (de Ziegler et al., 1991) or with a ‘straight-one oestrogen dose’ regimen (Lelaidier et al., 1992). The elapsed time between initiation of progesterone treatment and embryo transfer is important for successful nidation. The window for embryo transfer begins 48 h after starting progesterone administration and lasts for at least 4 days (Abdalla et al., 1997; Prapas et al., 1998).

Transfer of frozen–thawed pronucleated oocytes in stimulated cycles requires monitoring of follicular growth and ovulation induction with human chorionic gonadotrophin (HCG). Between two and four visits for gonadotrophin dose adjustment and timing of HCG ovulation induction are necessary. This procedure is time consuming and, therefore, expensive. In patients with irregular cycles or amenorrhoea, even gonadotrophin stimulation is frequently necessary to achieve ovulatory function. There is also a risk of multiple pregnancies in ovulatory cycles because additional in-vivo pregnancies added to the ones obtained after replacement of thawed zygotes can occur. Moreover, transfer of frozen–thawed PN in stimulated cycles showed unacceptably low pregnancy rates ≤17% (Al-Hasani et al., 1996, Table I). Thus we were looking for an effective, simple, convenient and inexpensive protocol for transfer of cryopreserved PN. We preferred a protocol with pure natural hormones such as 17β-oestradiol and progesterone in preparing for the embryo transfer required in artificial reproductive techniques.

Successful embryo transfer after oocyte donation in a patient treated with oestrogen–gestagen supplementation for primary ovarian failure was first reported in 1984 (Lutjen et al., 1984). Since then, transdermal sex hormone treatment has become available for women (Chetkowski et al., 1986) and men (Bals-Pratsch
Table I. Comparative pregnancy data for transfer of cryopreserved pronucleated oocytes in clomifene- or gonadotrophin-stimulated and artificial cycles. (Estraderm TTS100/Crinone 8%)

<table>
<thead>
<tr>
<th>Cycle (n)</th>
<th>Ovarian stimulation with clomifene citrate</th>
<th>Ovarian stimulation with HMG or FSH</th>
<th>Artificial cycle (Estraderm TTS100/ Crinone 8%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>8 (8)</td>
<td>7 (12.5)</td>
<td>8 (34.8)</td>
</tr>
<tr>
<td>56</td>
<td>5.5 (7/127)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>18.5 (10/54)†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HCG = human chorionic gonadotrophin; FSH = follicle stimulating hormone.
†Ongoing twin pregnancy (n = 1) and vanishing twin (n = 1).

et al., 1986). Transdermal therapeutic systems (TTS) are widely used because they mimic the normal physiological hormonal profile without the first pass effect of the liver. Thus possible negative effects of the supplemented hormone on metabolism can be reduced. Recently, a TTS-like progesterone delivering bioadhesive system for the vaginal wall was developed which provides a sustained-release effect (Wyeth-Ayerst, 1996). This preparation also allows targeted drug delivery, i.e. it maximizes the therapeutic effect of a drug by high local concentrations and minimizes the potential for systemic side-effects resulting from low blood concentrations. Buletti et al. demonstrated the ‘first uterine pass effect’ for transvaginal progesterone treatment (Buletti et al., 1997). This may improve embryo transportation in the genital tract and support embryo nidation by preventing abnormal uterine peristaltic contractions.

Materials and methods

The first 25 consecutive patients (aged 26–40 years, median 31 years) prepared for embryo transfer with transdermal oestradiol (Estraderm TTS 100®; Novartis, Wehr, Germany) and vaginal progesterone gel (Crinone 8%; Wyeth, Münster, Germany) similar to a previously described protocol (Gibbons et al., 1998; Figure 1) were studied. All women had regular cycles. However, down-regulation with a gonadotrophin-releasing hormone (GnRH) agonist was not performed. One to four Estraderm patches (each delivering 100 μg/24 h) were worn from cycle day 1 and were changed every other day in the evening on the following schedule: days 1–6, one TTS; days 7–10, two TTS; days 11–14, four TTS; days 15–18, three TTS; days 19–29, two TTS (de Ziegler et al., 1991). Crinone was administered daily in the morning from days 15–29 with a disposable plastic applicator containing 90 mg progesterone in a gel preparation. On cycle day 16, Crinone was exceptionally given in the evening of the day before embryo transfer and on cycle day 17 immediately after embryo transfer. A pregnancy test was performed on cycle day 29. If the test was positive, oestrogen and progesterone supplementation with Estraderm TTS and Crinone was continued for another 8 weeks because a corpus luteum was absent.
17β-oestradiol and progesterone in artificial ART cycles

Figure 1. Estraderm TTS100/Crinone 8% protocol for embryo transfer after thawing of cryopreserved zygotes. P = one application of vaginal progesterone gel (90 mg/day) from cycle days 15–28.

One hormonal assessment for oestradiol, luteinizing hormone (LH) and progesterone with commercial reagents (competitive enzyme-immunoassay SR1, automatic measuring apparatus SR1; Serono Diagnostics, Freiburg, Germany) and one transvaginal pelvic ultrasound examination (6.5 MHz vaginal probe, Sonoscope 30; Kranzbühler, Solingen, Germany) were carried out on cycle day 14. Embryo transfer was scheduled for day 17 if anovulation was confirmed by progesterone <3 ng/ml, absence of follicular growth and an endometrial thickness of at least 6 mm with the typical periovulatory triple line endometrium (Bakos et al., 1993). We employed previously described freezing and thawing procedures for pronucleated oocytes (Al-Hasani et al., 1996). Pre-embryo quality was scored from one to three according to the morphological condition of the cleaving pre-embryos, as ‘irregular’, ‘good’ and ‘ideal’ respectively (modified grading according to Veeck, 1991). The morphological grade of the embryo was then multiplied by the number of blastomeres to produce a quality score for each embryo. The score of all embryos transferred per patient were summed to obtain the cumulative embryo score (CES; Steer et al., 1992).

All patients had cryopreserved supernumerary PN from preceding IVF/intracytoplasmic sperm injection (ICSI) cycles. The duration of infertility was 1–14 years (median 4). ICSI was carried out as a result of male (n = 4), tubal (n = 6) or idiopathic infertility (n = 4). Four patients had ICSI using frozen–thawed epididymal (n = 1) or testicular spermatozoa (n = 3), because of obstructive (n = 1), non-obstructive (n = 1) azoospermia or anejaculation (n = 2). PN were cryopreserved according to a published procedure (Al-Hasani et al., 1996), thawed on cycle day 16 and transferred as cleaved embryos on day 17 after culture overnight.
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Table II. Endocrine data and thickness of endometrium by transvaginal ultrasound on cycle day 14 in 25 women

<table>
<thead>
<tr>
<th></th>
<th>Endometrial thickness (mm)</th>
<th>Oestradiol (pg/ml)</th>
<th>LH (mIU/ml)</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median</td>
<td>9</td>
<td>212</td>
<td>10.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Minimum</td>
<td>7</td>
<td>112</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Maximum</td>
<td>12</td>
<td>595</td>
<td>86.0</td>
<td>1.6a</td>
</tr>
</tbody>
</table>

aRelatively high value was due to increased production in the adrenal glands: follicular growth was absent and luteinizing hormone (LH) surge (13 IU/ml) was observed. It was not a sign of luteinization.

Table III. Cumulative pre-embryo quality score (morphological grade 1 to 3 for ‘irregular’, ‘good’ and ‘ideal’ multiplied by the number of blastomeres) for transferred embryos, and endometrial thickness in pregnant (n = 8) and non-pregnant subjects (n = 15)

<table>
<thead>
<tr>
<th></th>
<th>Cumulative pre-embryo score</th>
<th>Endometrial thickness (mm)</th>
<th>Oestradiol (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>17</td>
<td>9.5</td>
<td>218</td>
</tr>
<tr>
<td>Minimum</td>
<td>2</td>
<td>7.0</td>
<td>155</td>
</tr>
<tr>
<td>Maximum</td>
<td>29</td>
<td>12.0</td>
<td>321</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>9</td>
<td>9</td>
<td>212</td>
</tr>
<tr>
<td>Minimum</td>
<td>2</td>
<td>7</td>
<td>110</td>
</tr>
<tr>
<td>Maximum</td>
<td>34</td>
<td>11</td>
<td>595</td>
</tr>
</tbody>
</table>

Results

Embryo transfer was carried out in all 25 patients. Synoptic endocrine and ultrasound data are given in Table II. However, two patients had to be excluded from the evaluation. One patient did not administer Crinone on day 15 and 16 before the embryo transfer because of language problems. The other patient had a transfer with three PN without cleavage in culture overnight. One to three embryos were transferred. Two biochemical and six clinical pregnancies were achieved in the remaining 23 patients (Table I; overall pregnancy rate 34.8%, clinical pregnancy rate 26.1%). Biochemical pregnancies were defined as lowering of HCG prior to a gestational sac being seen by ultrasound examination. Synoptic data on the quality of transferred embryos (cumulative embryo score = CES) and the endometrial thickness for pregnant and non-pregnant subjects are shown in Table III. CES was higher in patients who became pregnant. One patient became pregnant with only one embryo transferred. Two patients had two gestational sacs in weeks 7 and 8, and both pregnancies are ongoing. However, one has become a singleton pregnancy after the disappearance of one twin. The overall implantation rate per embryo was 18.5% (Table I); and in the pregnant group (biochemical and clinical pregnancies; n = 10), it was 50.0% (10 implantations from 20 embryos transferred).
Table IV. Endocrine data from an intact pregnancy after transfer of frozen–thawed pronucleated human oocytes; microinjection using frozen–thawed testicular spermatozoa

<table>
<thead>
<tr>
<th>Week of gestation</th>
<th>Oestradiol (pg/ml)</th>
<th>Progesterone (ng/ml)</th>
<th>HCG (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2+0</td>
<td>250</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>2+3</td>
<td>395</td>
<td>8.0</td>
<td>0</td>
</tr>
<tr>
<td>4+1</td>
<td>384</td>
<td>12.8</td>
<td>43</td>
</tr>
<tr>
<td>5+5</td>
<td>151</td>
<td>6.5</td>
<td>4830</td>
</tr>
<tr>
<td>7+5</td>
<td>n.a.</td>
<td>n.a.</td>
<td>126 000</td>
</tr>
<tr>
<td>10+1</td>
<td>2761</td>
<td>38.0</td>
<td>285 000</td>
</tr>
</tbody>
</table>

HCG = human chorionic gonadotrophin; n.a. = not available.

Two pregnancies were even achieved with frozen–thawed PN after IVF/ICSI with frozen–thawed testicular spermatozoa. While one of these pregnancies is ongoing (endocrine data are given in Table IV), the other was completely aborted in gestational week 7. This miscarriage was preceded by heavy vaginal bleeding and accompanied by a decline of serum progesterone concentrations (Figure 2). Supplementation (i.m.) with hydroxyprogesterone caproate in combination with oestradiol valerate (Gravibinon®, Schering, Berlin, Germany) was given on days 22 and 24 after embryo transfer.

Discussion

Continuous oestrogen treatment is effective in preventing follicular development if treatment is started at the beginning of a cycle (de Ziegler et al., 1991). The increase in exogenous oestradiol doses in order to mimic the physiological pre-ovulatory pattern triggers the midcycle LH surge. The LH peak depends on an
individual oestradiol threshold concentration. However, this midcycle LH increase does not lead to ovulation or increase in progesterone (Lelaidier et al., 1992, 1995). We observed a midcycle LH peak (>7 IU/l, median 13 IU/l) in 16 of 25 patients (64%) without endocrine or sonographic findings of follicular growth or ovulation.

Endometrium preparation with exogenous oestradiol alone, in contrast to stimulation protocols or exogenous oestradiol after down-regulation with expensive GnRH agonists in cyclic women, has the advantages that it is convenient for the patients (e.g. embryo transfer can be scheduled ~2 weeks in advance) and is inexpensive. Only one visit for documentation of anovulation is required before embryo transfer. The possibility of cycle irregularities due to long-term effects of GnRH analogues does not exist.

In artificial cycles, transfer of frozen-thawed pronucleated oocytes is usually performed on day 2 or 3 of progesterone supplementation, which is started on cycle day 14 or 15 in addition to oestrogen treatment. There are three different effective routes available for progesterone replacement: oral, i.m. and vaginal. Although oral micronized progesterone is effective at higher doses in assisted reproductive technologies (Pouly et al., 1996), disadvantages of this therapy are variable absorption, metabolism by the liver (first pass effect) and possible central nervous system sedation by inactive metabolites. i.m. injections of progesterone are a safe and effective treatment (Miles et al., 1994; Queenan et al., 1997), but the repeated injections for several weeks are uncomfortable for the patients. Vaginal suppositories with progesterone dissolved in a petroleum jelly base or capsules with micronized progesterone in oil administered three times daily are effective in embryo transfer cycles (Schmidt et al., 1989; de Ziegler et al., 1991; Smitz et al., 1992). However, many women complain that these preparations result in messy vaginal secretions. The new bioadhesive vaginal gel Crinone 8% has the advantages that no messy vaginal secretions occur, that it has to be administered only once a day in the morning and that a daily dose of only 90 mg progesterone is sufficient for assisted reproductive cycles. The efficacy of this treatment regimen has also been proved by endometrial biopsies which showed a secretory ‘in phase’ transformation of the endometrium (Fanchin et al., 1997; Gibbons et al., 1998).

Progesterone is not only essential for endometrial receptivity in secretory transformation (Prapas et al., 1998). It also seems to play a crucial role in uterine contractions and may thus be the prerequisite for nidation of a transferred embryo. de Ziegler et al. examined the frequency and origin of uterine contractions (de Ziegler et al., 1996). Crinone applied every other day in oestrogenized women decreased and co-ordinated uterine contractions of antegrade direction at the cervix and of retrograde direction at the fundus from cycle days 15–20. Thus, we wanted to have high uterine progesterone concentrations around the time of embryo transfer and decided to apply Crinone in the evening of the day before and immediately after embryo transfer.

Pregnancy rates after transfer of cryopreserved pronucleated oocytes have been reported to be 7.7–27% (Al-Hasani and Ludwig, 1996).
first 23 patients treated with the TTS/Crinone-protocol resulted in an overall pregnancy rate as high as 34.8% and an overall implantation rate of 18.5%. The results underline the high clinical efficacy of replacement therapy with Crinone. To our knowledge, there is no other publication with higher pregnancy and implantation rates with frozen–thawed PN. In two cases, the PN resulted from ICSI with frozen–thawed testicular spermatozoa, which has not been reported before.

Progesterone therapy with Crinone in combination with Estraderm TTS combines the advantages of physiological treatment with natural hormones, and targeted drug delivery with the uterine first pass effect by the transvaginal application of the bioadhesive gel containing micronized progesterone without the first pass of the liver. Compared to other protocols for transfer of cryopreserved pronucleated oocytes, the Estraderm/Crinone scheme seems to be superior in achieving pregnancies. However, the transvaginal route of progesterone replacement may not be effective in patients with vaginal bleeding during early pregnancy after embryo transfer in artificial oestrogen–gestagen supplemented cycles. In these rare cases with possible reduced absorption of progesterone through the vaginal wall, we recommend further substitution therapy with i.m. progesterone until the bleeding has stopped.

The data presented here are preliminary. Comparisons between pregnancy rates in clomiphene- or gonadotrophin-stimulated cycles and the artificial cycles with Estraderm/Crinone (Table I) show the advantage of the artificial cycles, although these comparative data are retrospective. Ideally, a prospective, randomized, controlled trial should be performed to confirm our findings.

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