J Assist Reprod Genet (2012) 29:679–685
DOI 10.1007/s10815-012-9758-2

TECHNOLOGICAL INNOVATIONS

Hyaluronan-enriched transfer medium improves outcome in patients with multiple embryo transfer failures

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Received: 2 November 2011 / Accepted: 26 March 2012 / Published online: 12 April 2012

Abstract
Purpose To ascertain whether the use of hyaluronan-enriched transfer medium (HETM) improves pregnancy and implantation rates among embryo transfer patients with a history of multiple implantation failures.
Methods Patients (n=314) under the age of 40 and with a history of multiple unsuccessful embryo transfers were enrolled. There were three groups of patients: those undergoing fresh embryo transfer (fresh ET [n=111]), those undergoing vitrified-warmed ET in the natural cycle (WET-N [n=101]) and those undergoing WET in a hormone replacement cycle (WET-H [n=102]). On the day of ET, patients were randomized to HETM (0.5 mg/ml hyaluronan) or control medium containing no hyaluronan. Only patients with good quality embryos on day 3 were included.
Results For all three patients groups (fresh ET, WET-N and WET-H) pregnancy rates (37.5 %, 31.4 % and 41.2 %, respectively) were significantly higher when using HETM compared with control medium (10.9 %, 10.0 % and 15.7 %, respectively; p<0.05), and implantation rates when using HETM were also significantly higher compared with control medium (p<0.05). Miscarriage rates were similar in both groups.
Conclusion HETM significantly increased pregnancy and implantation rates among embryo transfer patients with a history of multiple unsuccessful implantations–regardless of method used to prepare the endometrium.

Keywords Hyaluronan · Vitrified-warmed embryo · Multiple embryo transfer failures · Implantation · Natural cycle · Hormone replacement cycle

Capsule HETM significantly increased pregnancy and implantation rates among patients with a history of multiple unsuccessful implantations–regardless of method used to prepare the endometrium.

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Introduction

Hyaluronan (HA) is a naturally existing macromolecule and a member of the glycosaminoglycan family that is abundant in human fluid secretions and the extracellular matrix [1]. HA is a major glycosaminoglycan in uterine fluid; it has been shown to increase cell-cell and cell-matrix adhesion, and may improve embryo apposition and attachment. It has been shown that HA biosynthesis increases dramatically in mice at the time of embryo implantation, and decreases to near-basal levels by the next day [2]. These findings suggest that HA may play a physiological role in implantation.

By binding to receptor CD44, HA may have cell-mediated activity [3]. Interestingly, human embryos possess CD44 receptors and these can be detected until the blastocyst has formed [3]. On the other hand, the human endometrium is receptive for implantation of an embryo only during a short secretory phase, the so-called implantation window. It has been shown that HA and its receptor (CD44) are present in the uterine endometrium, with the most abundant expression at the time of implantation [4]. Thus, both human embryo and uterine endometrium express the receptor for HA, and it is plausible that HA is involved in the initial phases of blastocyst attachment to the uterine endometrium.

Optimal management of infertility patients with a history of multiple implantation failures despite transferring good quality embryos remains unclear. Several approaches have

been attempted, such as changing the ovarian stimulation protocol, performing vitrified-warmed (as opposed to fresh) embryo transfer, or increasing the number of embryos transferred. However, these methods are liable to fail among patients with multiple embryo transfer failures, on the basis that impaired interaction between the embryo and endometrium might be an underlying problem. Urman and co-workers demonstrated that the use of HA-enriched transfer medium (HETM) increased implantation and clinical pregnancy rates, both in general and among women with previous implantation failures [5].

The implantation rate was shown to be increased in women receiving either cleavage- stage embryos or blastocysts [5]. However, the effectiveness of HETM is controversial. Two successive studies published in 2006 and 2007 were contradictory: one showed HETM to be effective (increasing implantation and pregnancy rates), while the other reported no such benefits [6, 7]. However in both of these studies, patients did not have a history of multiple implantation failures. Even in the report of Urman and co-workers, a history of previous implantation failures was not required for participation in the study [5].

This study was designed to ascertain whether the use of HETM (EmbryoGlue, Vitrolife, Sweden) improves pregnancy and implantation rates among patients with a history of multiple (four or more) unsuccessful embryo transfers.

Materials and methods

Patients

A total of 314 patients who were undergoing assisted reproductive technology (ART) treatment at the division of Reproductive Medicine in the Sugiyama clinic between March 2010 and September 2011 were enrolled for the present study. All patients were eligible for embryo transfer, under the age of 40, and had a history of four or more unsuccessful embryo transfers despite the use of morphologically good embryos (MGE). The uterine condition was checked for all patients by transvaginal ultrasound, hysperosalpingography, and hysterofiberscopy before beginning ART treatment, and it was confirmed that none of the patients had uterine fibroma which affected implantation, endometrial polyp, or hydrosalpix. If a patient showed at least one of these uterine abnormal conditions, she was excluded from the study. Signed informed consent was obtained from all patients, and this study was approved by the Institutional Board of the Sugiyama Clinic.

One hundred and eleven patients were undergoing fresh embryo transfer (fresh ET) with one or two MGE. These patients were randomized on the day of ET to HETM (transfer medium containing 0.5 mg/ml hyaluronan) or control transfer medium not containing hyaluronan (HUCUM, Nipro, Osaka; containing 7.5 % human serum albumin) by simple randomization method using patients' identification number as follows; patients were assigned to the treatment groups when their identification number was odd, and to the control groups when it was even.

The remaining 203 patients were undergoing vitrified-warmed embryo transfer with one or two MGE: 102 within the natural cycle (WET-N) and 101 within a hormone replacement cycle (WET-H). These patients were randomized to either HETM or control on the day of ET using simple randomization method as mentioned above. There was no patient who was undergoing fresh or vitrified-warmed ET without using MGE in this present study, because insufficient embryo quality itself was one of main causes of implantation failure.

Ovarian stimulation and IVF/ICSI procedure

All patients (n=314) were stimulated using either a mild stimulation protocol or a gonadotropin-releasing hormone antagonist (GnRH-ant) protocol. The mild stimulation protocol has been described elsewhere [8]; a brief description follows. The mild stimulation protocol consisted of: 50 mg clomiphene citrate (Serophene, Merck-Serono, Tokyo, Japan) per day for 5 days (3rd to the 7th day of the patient's menstrual cycle); 150 International Units (IU) of recombinant follicle-stimulating hormone (rec-FSH; Follistim, MSD, Tokyo, Japan) on days 3, 5 and 7 of the menstrual cycle; and an additional 150 IU per day of rec-FSH could be administered according to follicular growth. For ovarian stimulation using the GnRH-ant protocol, 150 IU of rec-FSH was administered each day, starting on the 3rd day of the menstrual cycle, and 0.25 mg per day of GnRH ant (Cetrotide, Shionogi, Osaka, Japan) was started when the dominant follicle reached 14 mm in diameter. Both agents (rec-FSH and GnRH-ant) were administered until the day of human chorionic gonadotropin injection (hCG; HCG injection, Fuji Pharma, Tokyo). Regardless of ovarian stimulation protocol, when dominant follicles reached ≥17 mm in diameter, 10,000 IU of hCG was administrated, and oocyte retrieval was performed 35 h afterwards.

The IVF procedure used in this study has been described previously [9]. Oocytes were retrieved transvaginally using a needle-guided technique, aided by ultrasonography. All follicles with a diameter >15 mm were aspirated individually, using a 19-gauge needle connected to a tube for suction. The needle was removed after the aspiration of each follicle. All follicles were washed by culture media. Semen was produced by masturbation and, after washing, motile sperm were separated using a 30–60 min swim-up period. In vitro insemination was performed by incubating each oocyte with $50–100\times10^3$ motile sperm within 5–6 h of collection.

In vitro insemination was not performed when there was evidence of male-factor infertility. In these cases, intracytoplasmic sperm injection (ICSI) was performed according to a previous report [10]. Oocytes were examined using a dissecting microscope 16–18 h after either insemination or ICSI. The presence of two pronuclei with extrusion of the second polar body was taken as evidence of successful fertilization. All embryos were cultured in culture medium (HUCUM ,Nipro, Osaka, Japan) for 72 h after insemination or ICSI, and embryos were examined microscopically 72 h after insemination or ICSI.

Preparation of HETM and embryo transfer

A commercial available medium (Embryo Glue®, Vitrolife, Sweden) is used as HETM. On the day before ET, 1.0–2.0 ml of Embryo Glue® was added to the dish and equilibrated in 37 °C and 6 % CO_2, 5 % O_2 and 89 % N_2 for 4–18 h.

On the day of transfer, embryos were placed in the well containing pre-equilibrated HETM for 10–30 min under 37 °C and 6 % CO_2, 5 % O_2 and 89 % N_2 before ET. The transfer catheter (φcon ET catheter, Kitazato Supply, Shizuoka) was firmly attached with a pre-rinsed syringe and approximately 0.5 ml of HETM was expelled to the syringe. Under the microscope, the embryos were gently loaded into the catheter in approximately 5–10 μL of HETM and a small volume of air was followed. The embryos with HETM were slowly withdrawn the catheter which was rinsed with pre-equilibrated HETM while maintaining the steady pressure on the plunge of the syringe.

Embryos meeting the following criteria were defined as MGE: developed to at least the 7-cell stage with less than 10 % fragmentation within 72 h after either insemination or ICSI. This system of embryo assessment was based on the classification system described by Veeck [11]. Embryos were replaced into the patient's uterus transcervically using a soft catheter (Kitazato ET catheter, Kitazato Supply, Shizuoka, Japan). In all patients, either one or two embryos were transferred [12].

In the fresh ET group, ET was performed on day 3. Any remaining embryos were cryopreserved using vitrification [13]. On days 4 and 8 following ET, 3,000 IU hCG was injected for luteal support. A combination of estrogen and progesterone was administered orally for 10 days after ET. For patients at elevated risk of developing ovarian hyperstimulation syndrome, hydroxyprogesterone caproate was administered instead of hCG.

In the WET-N group, the day of embryo transfer was defined as 3 days after the ovulation. For patients with regular menstrual cycles, ET was performed in the natural cycle; neither clomiphene citrate nor human menopausal gonadotropins were administered to stimulate follicle growth and, hCG was not administered to induce ovulation. The day of ovulation was confirmed by serum luteinizing hormone (LH), estradiol (E2) and progesterone levels. The same luteal support as in the fresh ET group was administered. In the WET-H group, the uterine endometrium was prepared for ET using conjugated estrogens (Premarin 0.625 mg, Wyeth, Tokyo, Japan) and transdermal estradiol (Estrana TAPE 0.72 mg, Hisamitsu Pharmaceutical, Tokyo, Japan). These treatments were administered from the 3rd day of the menstrual cycle (1st day without bleeding) until the day of the urinary pregnancy test. Administration of progesterone (100 mg in oil; Progestone Depot-S, Fuji Pharmaceutical, Tokyo, Japan) was initiated on the 12th day of the menstrual cycle. Three days after initiation of progesterone treatment, embryos were warmed and those that had survived were used in embryo transfer.

Assessment and data analysis

A pregnancy was recognized when the development of a gestational sac was detected by transvaginal ultrasound on the 21st day after: oocyte retrieval in the fresh ET group, ovulation in the WET-N or initial administration of progesterone in the WET-H group, respectively. Pregnancy rate was calculated as the number of the patients with confirmed gestational sac by transvaginal ultrasound divided by the number of treated patients, and implantation rate was calculated as the number of geastational sacs divided by the number of transferred embryos. The fetal heart beat (FHB) rate was calculated as the number of patients with confirmed FHB by transvaginal ultrasound at 7 weeks of gestation divided by the number of treated patients. Miscarriage was defined as no cardiac activity at 7 weeks of gestation or loss of pregnancy before 22 weeks of gestation.

Rates of pregnancy, implantation FHB and miscarriage were compared between the two transfer media (i.e. HTEM vs. Control) in three patient groups. Statistical analysis was performed using an unpaired-t test and a Chi-square test. Statistical significance was set at $p<0.05$.

Results

Patient characteristics and outcomes for the fresh ET group are summarized in Table 1. The average age and number of previous embryo transfer failures were similar among both HETM and control medium patients. The numbers of embryos transferred was also similar for the two transfer media. The proportion of single embryo transfer (SET) in the HETM group was significantly higher than that in the control group ($p=0.0256$). And, both the clinical pregnancy rate and the implantation rate were significantly higher with HETM than with control medium ($p=0.0011$ in pregnancy

Table 1 Patients characteristics and outcomes of patients in the fresh embryo transfer (fresh ET) group

	HETM	Control	p-value
Number of patients	56	55	–
Age (years)*	37.8±0.4	38.0±0.4	0.7844
Previous ET attempts*	5.9±0.4	6.0±0.4	0.8912
IVF/ICSI	36/20	31/24	0.7291
Number of transferred embryos*	1.5±0.2	1.5±0.2	0.9131
Proportion of SET (%)	51.7	30.9	0.0256
Pregnancy rate (%)	37.5 (21/56)	10.9 (6/55)	0.0011
Implantation rate (%)	27.4 (23/84)	7.3 (6/82)	0.0007
FHB rate (%)	30.4 (17/56)	9.1 (5/55)	0.0050
Miscarriage rate (%)	19.0 (4/21)	16.7(1/6)	0.8947

ET embryo transfer, *HETM* hyaluronan-enriched transfer medium, *SET* single embryo transfer, *FHB* fetal heart beat; *mean ± standard error of the mean (SEM)

rate and $p=0.0007$ in implantation rate, respectively). FHB rate in the HETM was significantly higher than that in the control group ($p=0.0050$). There was no significant difference in miscarriage rate.

Patient characteristics and outcomes for the WET-N group are summarized in Table 2. The average age and number of previous embryo transfer failures were similar among both HETM and control medium patients. The number of embryos transferred was also similar between the groups but the proportion of SET in the HETM group was higher than that in the control group, even though there was not a significant difference between the groups ($p=0.086$). However, both the clinical pregnancy rate and implantation rate were significantly higher with HETM versus control medium ($p=0.0081$ in pregnancy rate and $p=0.0068$ in implantation rate, respectively). FHB rate in the HETM was significantly higher than that in the control group ($p=0.0412$), and no significant difference in miscarriage rate between the groups was observed.

In table 3, patient characteristics and outcomes for the WET-H group are summarized in the other groups. The proportion of SET in both HETM and control groups was similar. The pregnancy ($p=0.0043$), implantation ($p=0.0048$) and FHB rates ($p=0.0126$) were significantly higher with HETM than with control medium but there was no significant difference in miscarriage rate. All patients in both WET-N and WET-H groups received elective embryo transfers, because suitable embryos were selected before cryopreservation. But 89.3 % of HETM and 94.5 % of control groups for fresh ETs were performed using elective embryo transfers.

Discussion

Implantation is delicate process involving complex interactions of factors derived from either the embryo or endometrium [14, 15]. Over the years, significant improvement in ART success rates has been achieved. However, embryo implantation still remains a major limiting factor. In an animal model of implantation, Gardner et al. found significant increases in both implantation and fetal development rates when HA was present in the transfer medium as the only macromolecule, compared with transfer medium containing no macromolecules [16]. HA may also have a role in the preparation of the endometrium for embryo implantation. Its level increases significantly on the day of implantation in mouse uterus, particularly in regions that contain

Table 2 Patients characteristics and outcomes of patients in the vitrified-warmed embryo transfer in natural cycle (WET-N) group

	HETM	Control	p-value
Number of patients	51	50	–
Age (years)[a]	36.3±0.3	36.9±0.4	0.8911
Previous ET attempts[a]	5.8±0.4	6.0±0.4	0.8121
IVF/ICSI	29/22	22/28	0.1961
Number of transferred embryos[a]	1.6±0.2	1.5±0.2	0.9112
Proportion of SET (%)	35.3	20.0	0.0860
Pregnancy rate (%)	31.4 (16/51)	10.0 (5/50)	0.0081
Implantation rate (%)	22.0 (18/82)	6.7 (5/75)	0.0068
FHB rate (%)	19.6 (10/51)	6.0 (3/50)	0.0412
Miscarriage rate (%)	37.5(6/16)	40.0 (2/5)	0.9200

ET embryo transfer, *HETM* hyaluronan-enriched transfer medium, *SET* single embryo transfer, *FHB* fetal heart beat; [a]mean ± standard error of the mean (SEM)

Table 3 Patients characteristics and outcomes of patients in the vitrified-warmed embryo transfer in hormone replacement cycle (WET-H) group

	HETM	Control	p-value
Number of patients	51	51	–
Age (years)[a]	36.7±0.3	36.8±0.3	0.8831
Previous ET attempts[a]	6.2±0.4	6.1±0.4	0.8591
IVF/ICSI	33/18	31/20	0.6821
Number of transferred embryos[a]	1.5±0.2	1.6±0.2	0.8912
Proportion of SET (%)	17.6	17.6	–
Pregnancy rate (%)	41.2 (21/51)	15.7(8/51)	0.0043
Implantation rate (%)	27.3 (21/77)	9.9 (8/81)	0.0048
FHB rate (%)	29.4 (15/51)	9.8 (5/51)	0.0126
Miscarriage rate (%)	28.6 (6/21)	37.5 (3/8)	0.6423

ET embryo transfer; *HETM* hyaluronan-enriched transfer medium; *SET* single embryo transfer; *FHB* fetal heart beat; [a]mean ± standard error of the mean (SEM)

stromal cells proliferating in preparation for embryo implantation [2]. Based on these data, HETM became a candidate for improving implantation rates.

The use of HA in transfer medium may offer several advantages in the implantation process. Concern has been expressed regarding immediate or late expulsion of embryos after their transfer to the uterine cavity [17]. However, the physical properties of HA prohibit the expulsion of embryos from the uterine cavity after transfer. It has also been suggested that the use of HA in transfer medium can facilitate its dispersal within uterine fluid, thereby facilitating transport of the embryo to the endometrium [16]. Moreover, it has been shown that, as well as proteoglycans, HA is involved in cell-cell and cell-matrix adhesion, and in activation or inhibition of protease [1]. A role for HA in embryo adhesion is also suggested by the observation that tumors are highly enriched with HA and that their invasiveness is correlated with HA expression [18, 19]. These data and the detection of HA receptors on mammalian embryos suggest that HA plays a role in an embryo attachment, implantation, and development after being transferred to the uterine cavity [3].

Many clinicians have attempted to evaluate the effectiveness of HETM in ART patients. Some have reported improved pregnancy and implantation rates with HETM, while others have reported no such advantage. Nevertheless, a recent Cochrane review demonstrated improved pregnancy and implantation rates when high concentrations of HA are used [20]. Also, one study [21] demonstrated that the use of HETM is beneficial for patients with a history of multiple implantation failures undergoing fresh cleavage-stage embryo transfer. It is possible that patient selection might explain the differences between study outcomes. Human embryos possess HA receptor (CD4) and these can be detected between early-cleavage stage embryo and blastocyst [3]. On the other hand, the human endometrium is receptive for implantation of an embryo only during the implantation window opened and it has been shown that HA receptors (CD44) are present in the uterine endometrium, with the most abundant expression at the time of implantation [4]. Thus, both human embryos and the uterine endometrium express the receptor for HA, and cleavage-stage embryos binding HA might facilitate attachment to the uterine endometrium until embryos grow to the hached blastocyst. We speculate that it is plausible that HETM is effective for the cleavage-stage embryos.

This present study focused on the endometrial preparation for vitrified-warmed embryo transfer. This kind of approach has not been explored in previous studies. The study by Hambiliki et al. was limited to the transfer of cleavage-stage frozen-thawed embryos, and it compared the results of using high versus low concentration of HA in the transfer media. The results suggested that high concentration of HA supports the embryo during initial implantation into the endometrium [22]. However, that study did not show a difference in clinical pregnancy and implantation rates according to the difference of endometrial preparation for frozen-thawed embryo transfer and it lacked information about endometrial preparation. On the other hand, the impact of endometrial preparation in combination with HETM on clinical results was clearly demonstrated in this study, and moreover, all embryos used in it were not cryopreserved by slow freezing method but rather vitrified and warmed. Therefore, this study will help guide us in clinical decisions particularity for vitrified embryos.

Many factors may influence the efficacy of embryo attachment or implantation into the uterine endometrium. These include embryo quality, patient age, endometrial thickness and several uterine factors such as fibroma, endometrial polyp and uterine abnormalities. Since the uterine conditions which affect implantation were checked, none of the patients in the study had the uterine factors described above. Embryo quality is known to be important, but some patients have never achieved pregnancy despite having morphologically good embryos for every transfer, and having adequate endometrial thickness. One possible explanation for implantation failure is that embryos carrying chromosomal abnormalities might be included in the morphologically

optimal embryo groups. Indeed, according to one study, 20.9 % of cleavage stage embryos which derived from patients who were more than 36 years of age and had a history of multiple implantation failures showed euploidy according to the results of pre-implantation genetic diagnosis (PGD) [23]. Viewed from the opposite side, about 80 % of the embryos were aneuploidy. Still, the study by Magi et al. also indicated that the incidence of chromosomal abnormalities was significantly lower at the 7–8 cell stage compared to all other stages [23]. In this present study, only morphologically good embryos, which developed to at least the 7-cell stage with less than 10 % fragmentation within 72 h after either insemination or ICSI, were used for the embryo transfer. From the report by Magi et al, 50 % of the embryo that we used for ET might be included chromosomally normal embryos, and a history of multiple implantation failures (≥4 times) could not attribute to embryo chromosomal abnormalities. In our opinion, the key to solving this problem might be HA, and the use of HETM might facilitate successful implantation in these patients.

We selected patients who were below the age of 40, with a history of four or more unsuccessful embryo transfers and with one or two morphologically good embryos for embryo transfer. With this approach, the influence of maternal age should be minimized. The history of multiple embryo transfer failures, despite morphologically good embryos, served to maximize the likelihood of HA having an effect. Previous reports did not consider the preparation of endometrium for embryo transfer, therefore results from fresh embryo transfers were mixed with those from frozen-thawed embryo transfers (both natural cycle and hormone replacement cycle). In the present study, we separated patients according to the method used for preparing the endometrium. Compared with control medium, HETM significantly increased pregnancy and implantation rates regardless of the method used for preparing the endometrium.

Moreover, in the fresh ET group, the proportion of SET in the HETM group was significantly higher than that in the control group. Similarly, in the WET-N group, the proportion of SET in the HETM group was higher than in the control group, albeit not significantly. In general, one group with higher number of transferred embryos showed better pregnancy than the other group with smaller number of transferred embryos. From this fact, it was thought that a group showing higher proportion of SET was disadvantageous to the success of ART treatment. However, in this present study HETM group in fresh ET group showed higher pregnancy and implantation rates than those in the control group regardless of higher proportion of SET. This indicated that HETM was effective for implantation of embryos. Thus, inadequate levels of HA might explain some of our patients' history of four or more unsuccessful embryo transfers.

Forty-one women (n=41) for whom HETM was used became pregnant. At the latest follow up, 20 of these women had given birth to 25 health babies (13 boys and 12 girls), 14 were still pregnant and 7 had miscarried. We are now investigating the perinatal outcomes.

In conclusions, HETM significantly increased embryo transfer pregnancy and implantation rates compared with control transfer medium, among patients with a history of multiple unsuccessful embryo transfers. This difference was observed regardless of the method used to prepare the endometrium. For patients with multiple implantation failures despite morphologically good embryos and adequate endometrial thickness, the present data suggest that HETM as a possible solutions.

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