Original Article
Obstet Gynecol Sci 2016;59(3):201-207
http://dx.doi.org/10.5468/ogs.2016.59.3.201
pISSN 2287-8572 · eISSN 2287-8580

Obstetrics & Gynecology Science

Efficacy of hyaluronan-rich transfer medium on implantation and pregnancy rates in fresh and frozen-thawed blastocyst transfers in Korean women with previous implantation failure

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Objective
To evaluate the effect of hyaluronan-rich transfer medium on pregnancy and implantation rates in fresh and frozen-thawed embryo transfers in Korean women with previous implantation failure.

Methods
This retrospective study included 283 blastocyst transfers in patients with previous embryo transfer failure at a private fertility clinic. In the study group (n=88), blastocyst transfers were performed using an hyaluronan-rich transfer medium prior to transfer, whereas blastocyst transfers without any treatment served as controls (n=195). According to the type of transfer (fresh elective or frozen-thawed), all the blastocyst transfers were divided into two study and two control groups.

Results
The patient's mean age, serum anti-Müllerian hormone level, causes of infertility, embryo quality, and the number of transferred embryos were comparable between the study and control groups. There were no significant differences in clinical pregnancy rate (45.5% vs. 43.1%), implantation rate (28.9% vs. 28.8%), and clinical abortion rate (10.0% vs. 8.3%) between the two groups, and these findings were not changed after subgroup analysis according to the type of transfer.

Conclusion
The use of hyaluronan-rich transfer medium in the blastocyst transfer does not appear to have any significant effect on the implantation and pregnancy rates in patients with previous implantation failure.

Keywords: Blastocyst; Embryo transfer; Hyaluronic acid; Implantation; Pregnancy

Introduction

The success rates for *in vitro* fertilization (IVF) and embryo transfer (ET) in humans have increased because of improvements in ovulation induction medications, regimens, and culture technology [1]. Advances in human embryo culture technology have evolved continuously over the years with better culture media and culture systems [1-3]. These have led to an increase in blastocyst formation and pregnancy rates, but several factors that contribute to embryo development and implantation have not yet been determined [1,4,5]. Sequential media have taken into account the changes in embryo physiol-

Received: 2015.9.30. Revised: 2015.12.22. Accepted: 2015.12.29.
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ogy and nutritional requirements that occur during prolonged in vitro culture [3,6], and have progressed with optimization of amino acid, vitamin, and ionic composition, and more recently, with the addition of macromolecules [1-3,6]. Both in animal and human embryo culture systems, macromolecules such as albumin have traditionally been supplemented as a source of energy necessary for embryo development and as reservoirs for vitamins and minerals [5,7].

Hyaluronan (HA) is a naturally existing macromolecule, and is abundant in human fluid secretions and extracellular matrix in the female reproductive tract [4,8,9]. The addition of HA to culture media was shown to increase both embryo development and implantation in animals [10-12]. Several human studies have assessed the impact of HA-rich culture and transfer medium in ET on implantation and clinical pregnancy rates, but the results of these studies are inconsistent [4,5,7,13-15]. A recent Cochrane review demonstrated an improved clinical pregnancy rate when high concentrations of HA in transfer media were used [16].

The aim of this study was to evaluate the efficacy of HA-rich transfer medium in fresh and frozen-thawed blastocyst transfer on the implantation and pregnancy rates in Korean women with a history of previous implantation failure.

Materials and methods

1. Subjects

This retrospective case-control study was approved by the institutional review board of Maria Fertility Clinic. Unselected patients aged 26 to 44 years who had at least one episode of previous ET failure and underwent fresh cycle blastocyst transfer or frozen-thawed blastocyst transfer at Busan Maria Fertility Clinic were included in this study. Oocyte retrievals were performed after controlled ovarian hyperstimulation using a recombinant follicle-stimulating hormone (Gonal-F, Serono, Istanbul, Turkey; Follitrope, LG, Seoul, Korea), with a gonadotropin-releasing hormone agonist long protocol or a gonadotropin-releasing hormone antagonist short protocol, followed by conventional IVF or IVF via intracytoplasmic sperm injection. Basal serum anti-Müllerian hormone (AMH) level was measured using the AMH Gen II assay (Beckman Coulter Inc., Brea, CA, USA).

A total of 88 blastocyst transfers using a commercially available HA-rich transfer medium containing 0.5 mg/mL HA (EmbryoGlue, VitroLife, Kungsbacka, Sweden) between October 2012 and April 2014 were included in the study group, and 195 blastocyst transfers conducted without additional transfer media between October 2012 and April 2014 served as controls. All transfers were divided into two study groups and two control groups according to the type of transfer (fresh elective or frozen-thawed). Pregnancy was confirmed by a serum β-human chorionic gonadotropin (hCG) test using the ARCHITECT i4000SR (Abbott, Abbott Park, IL, USA) 9-10 days after blastocyst transfer. Blood samples were collected from all subjects in accordance with the Declaration of Helsinki guidelines, and both the intra- and inter-assay coefficients of variation for serum AMH and β-hCG were below 5.0%.

2. Procedures of embryo transfer

Embryo culture and cryopreservation in fresh and frozen-thawed cycles, and warming of blastocysts in frozen-thawed cycles, were performed as previously described elsewhere [17,18]. In fresh ET, good quality embryos were selected on the morning of day 5 by blastocyst grading criteria described by Gardner and Schoolcraft [19]. Blastocysts were then placed in a well containing pre-equilibrated HA-rich transfer medium for at least 30 minutes at 37°C in a 5% CO_2 environment prior to transfer in the study group; in the control group, blastocysts were transferred without using additional transfer medium.

In frozen-thawed ET, good quality embryos were selected in the same manner as in fresh ET. HA-rich transfer medium (0.5 mL) was added to the dish and equilibrated at 37°C in 5% CO_2 for 4 to 12 hours on the day before ET in the study group. Frozen embryos were transferred for 5 minutes to warming solution 1 containing 0.5 M sucrose dissolved in Dulbecco's phosphate buffered saline and supplemented with 20% human follicle fluid. The embryos were then transferred to warming solution 2 in Dulbecco's phosphate buffered saline supplemented with 20% human follicle fluid for 5 minutes. Embryos were then transferred into equilibrated blastocyst medium (Cook Medical, Brisbane, Australia), and cultured at 37°C in 5% CO_2. The post-thawing survival of the embryos was observed under an inverted microscope 3 to 24 hours after warming. In the study group, embryos were placed in a well containing pre-equilibrated HA-rich transfer medium for at least 30 minutes at 37°C in 5% CO_2 on the day of transfer.

3. Outcome measures related to pregnancy and implantation

Rates of clinical pregnancy, clinical abortion, and implantation were compared between study and control groups. Clinical pregnancy was defined in the present study when a gestational sac could be seen on ultrasonography examination. The clinical pregnancy rate was defined as the number of cases with evidence of at least one gestational sac divided by the number of transfers, the clinical abortion rate was defined as the number of clinical pregnancy losses including ectopic pregnancy before the 20th week of gestation divided by the total number of clinical pregnancies, and the implantation rate was defined as the number of gestational sacs confirmed by transvaginal ultrasonography divided by the number of embryos transferred [20].

4. Statistical analysis

All data are expressed as the mean±standard deviation. All statistical analyses were performed using PASW ver. 18.0 (SPSS Inc., Chicago, IL, USA). An unpaired *t*-test and a Mann-Whitney *U*-test were used for the comparison of the clinical parameters between study and control groups, and comparisons of the proportion of causes of infertility and embryo quality, implantation rate, clinical pregnancy rate, and clinical abortion rate between the two groups were performed using a chi-square test and a Fisher's exact test. *P*-values <0.05 were considered significant for all analyses.

Table 1. Baseline clinical parameters of the study and control groups

Parameter	Study group	Control group	*P*-value[a]
Total	(n=88)	(n=195)	
Age (yr)	35.7±3.9	36.1±4.0	0.402
Basal AMH levels (ng/mL)	4.5±3.7 (n=41)	4.5±3.8 (n=97)	0.962
Transferred embryos	1.89±0.41	1.91±0.34	0.648
Causes of infertility			
Male factor	6 (6.8)	18 (9.2)	0.500[b]
Tubal	15 (17.0)	37 (19.0)	0.698[b]
Endometriosis	7 (8.0)	19 (9.7)	0.630[b]
Ovulatory	26 (29.5)	56 (28.7)	0.887[b]
Others	10 (11.4)	16 (8.2)	0.394[b]
Unexplained	24 (27.3)	49 (25.1)	0.703[b]
ICSI use	121/166 (72.9)	290/372 (78.0)	0.201[b]
Embryo quality			
Grade A	25/166 (15.1)	64/372 (17.2)	0.536[b]
Grade B	93/166 (56.0)	220/372 (59.1)	0.499[b]
Grade C	48/166 (28.9)	88/372 (23.7)	0.195[b]
Fresh cycle	(n=32)	(n=91)	
Age (yr)	35.5±3.3	36.7±3.7	0.117
Basal AMH levels (ng/mL)	3.1±1.4 (n=17)	3.4±2.6 (n=40)	0.556[c]
Transferred embryos	1.9±0.3	2.0±0.3	0.121
Retried oocytes	17.8±8.1	17.6±7.6	0.880
Thawing embryo transfer	(n=56)	(n=104)	
Age (yr)	35.8±4.2	35.6±4.1	0.807
Basal AMH levels (ng/mL)	5.5±4.5 (n=24)	5.3±4.2 (n=57)	0.869[c]
Transferred embryos	1.9±0.5	1.8±0.4	0.496

Values are mean±standard deviation or number (%).
AMH, anti-Müllerian hormone; ICSI, intracytoplasmic sperm injection.
[a]Unpaired *t*-test; [b]Chi-square test; [c]Mann-Whitney *U*-test.

Table 2. Clinical outcomes after fresh and frozen-thawed blastocyst transfer in the study and control groups

Parameter	Study group	Control group	*P*-value[a]
Total			
Implantation rate	48/166 (28.9)	107/ 372 (28.8)	0.971
Clinical pregnancy rate	40/88 (45.5)	84/195 (43.1)	0.709
Clinical abortion rate	4 /40 (10.0)	7/84 (8.3)	0.745[b]
Fresh cycle			
Implantation rate	18/60 (30.0)	49/180 (27.2)	0.678
Clinical pregnancy rate	15/32 (46.9)	35/91 (38.5)	0.405
Clinical abortion rate	2/15 (13.3)	3/35 (8.6)	0.629[b]
Thawing embryo transfer			
Implantation rate	30/106 (28.3)	58/192 (30.2)	0.730
Clinical pregnancy rate	25/56 (44.6)	49/104 (47.1)	0.765
Clinical abortion rate	2/25 (8.0)	4/49 (8.2)	1.000[b]

Values are number (%).
[a]Chi-square test; [b]Fishers' exact test.

Results

Clinical parameters were similar between the study and control groups (Table 1). No significant difference in the number of transferred embryos or number of oocytes retrieved in the fresh cycles was found between the two groups. The proportions of causes of infertility and embryo quality were comparable between the two groups.

In Table 2, the implantation rate in the study group (28.9%) was not different from that of the control group (28.8%), and there were no statistically significant differences in the clinical pregnancy rate (45.5% vs. 43.1%, *P*=0.709) and clinical abortion rate (10.0% vs. 8.3%, *P*=0.745) between the two groups. In subgroup analysis according to the transfer type, no significant differences were found in the implantation rate between the study and control groups after fresh elective transfers (30.0% and 27.2%, respectively) or frozen-thawed transfers (28.3% and 30.2%, respectively). Neither the clinical pregnancy rate nor clinical abortion rate after fresh elective transfers or frozen-thawed transfers was different between the two groups.

Discussion

Implantation is one of the cardinal components of human reproduction, and successful implantation requires a functionally normal embryo at the blastocyst stage, a receptive endometrium, and communication between embryo and endometrium [21]. Optimal management of infertility patients with previous unsuccessful implantations in spite of transferring good quality embryos remains undetermined, suggesting that implantation remains a major limiting step in the success of human assisted reproductive technology [4,15]. Several approaches have been attempted, including changing the ovarian stimulation protocol, performing vitrified-warmed ET as opposed to fresh ET, or increasing the number of embryos transferred [15]. However, because impaired interaction between the embryo and endometrium might be an underlying problem, these methods are prone to fail in patients with multiple ET failures [9,15]. Thus, it is necessary to search for alternative approaches to improve implantation of human embryos in the IVF-ET procedure. In an attempt to optimize human in vitro embryo culture systems, supplementation of proteins which provide a source of energy for embryo development and act as reservoirs for vitamins and minerals has traditionally been performed [2,3]. Albumin, which is abundant in the female reproductive tract, is the most commonly used protein source for human in vitro embryo culture systems, and sources for culture media are the patient's own serum, commercially pooled human serum albumin (HSA), and recombinant human albumin [2,5,13,14].

Recently, HA supplementation in ET media has emerged as a promising option for the improvement of implantation and

pregnancy rates of embryos in human IVF-ET. Gardner et al. [10] reported that the highest rates of implantation and fetal development after blastocyst transfer in mice were observed when HA is supplemented as the macromolecule in culture media, and that these beneficial effects of HA were due to its presence in transfer medium. They concluded that embryo culture media should contain both serum albumin and HA, while the transfer medium need only contain HA. Simon et al. [7] suggested that HA can successfully replace albumin as a macromolecule in a human ET medium, and the use of HA would be an important step in the development of human embryo culture media free of blood-derived additives.

HA belongs to the glycosaminoglycan family, and is a high molecular weight linear polysaccharide comprised of alternating D-glucuronic acid and N-acetyl-D-glucosamine residues [14]. It is abundant in the human female cervix, oviduct, uterus, and ovarian follicle, and increases up to the time of implantation [14,22,23]. Gardner et al. [24] reported that embryos cultured to the blastocyst stage in HA-enriched sequential media are readily cryopreserved and maintain their viability after thaw.

The mechanism by which HA promotes implantation remains unresolved. It may be related to the initial stages of implantation, as HA is known to regulate several functions such as gene expression, signaling, proliferation, migration, adhesion, morphogenesis, and differentiation [20]. It is assumed to contribute to apposition and attachment of the blastocyst to the endometrium by increasing cell-to-cell and cell-to-matrix adhesion [25], and appears to be important for placentation in human reproduction. These actions are known to be mediated by the cell surface adhesion glycoprotein CD44 that is present in the uterine endometrium, with the most abundant expression at the time of implantation [15]. CD 44 is the principal receptor allowing binding of HA and entry into the cell for degradation by acid hydrolases [5,26]. Other proposed mechanisms by which HA acts on implantation [9] are by indirectly promoting angiogenesis [27] and/or by creating a highly viscous environment enabling human embryos to be easily included in uterine secretions [10].

The aim of the present study was to evaluate the efficacy of commercially available HA-rich transfer medium in fresh and frozen-thawed blastocyst transfer on the implantation and pregnancy rates in Korean women with a history of previous implantation failure. To the best of our knowledge, this study is the first to report in Korea on the efficacy of HA-rich transfer medium. We found no significant effect of HA-rich transfer medium on the implantation and pregnancy rates in patients with previous implantation failure.

It remains controversial whether the use of HA-rich transfer medium improves pregnancy and implantation rates among ET patients. Several randomized trials demonstrated the beneficial effect of HA-rich transfer medium on implantation and pregnancy [4,13-15,28]. Two large prospective trials demonstrated that the use of HA-enriched transfer medium increased implantation and clinical pregnancy rates [13,14], and another randomized trial reported that there were significantly higher implantation (16.3%) and clinical pregnancy rates (35.2%) in patients using HA enriched commercial ET medium compared to routine ET medium without HA (4.8% and 10.0%, respectively) [4]. A large prospective observational study demonstrated that the use of HA in the transfer media significantly increased the positive hCG and implantation rate without increasing the delivery rate [20]. On the contrary, two randomized [7,29] and one observational study [5] reported no significant benefit of HA-rich transfer medium on pregnancy and implantation rates. Loutradi et al. [5] conducted a retrospective, matched group study, and compared pregnancy rates between high HA/HSA ET medium and low HA/HSA medium groups. Total pregnancy rates per ET were similar in both groups (23.1% vs. 24.7%), and it is consistent with the result of our study. Korosec et al. [9] conducted a prospective randomized study including 279 single blastocyst transfers in women aged <37 years in their first, second, or third treatment cycle. They reported that overall pregnancy rates after fresh elective and frozen-thawed single blastocyst transfer were similar in both study and control groups. However, HA results in significantly higher pregnancy rates in a selected subgroup of women; those with ≥2 blastocysts developed to day 5 and a previous implantation failure (55% vs. 10% per transfer, P=0.012), and it contrasts with the result of ours. The Cochrane review [16] reported a higher clinical pregnancy rate in the high HA transfer media group (odds ratio [OR], 1.41; 95% confidence interval [CI], 1.22 to 1.63). In subgroup analysis, however, ETs on day 5 showed no evidence of a treatment effect (OR, 1.19; 95% CI, 0.67 to 2.09), and there was no evidence of a treatment effect in the participants exposed their embryos to HA for more than 10 minutes (OR, 1.29; 95% CI, 0.85 to 1.97) [16]. These results are partially consistent with ours. Another subgroup analysis showed increased clinical pregnancy rate in the poor prognosis group (OR, 4.53;

95% CI, 2.54 to 8.10), but this subgroup analysis could not be conducted in the present study because of the relatively small sample size of ours.

Our study had some considerable limitations mainly stem from its retrospective study design. We were unable to adjust some critical factors between study and control groups, such as ovarian stimulation protocol, dose of gonadotropin, and supplements during IVF-ET cycles. Additionally, there are several factors related to the efficacy of embryo attachment or implantation into the uterine endometrium, including patient age, embryo quality, and endometrial thickness, as well as several uterine factors, such as leiomyomas, polyps, and other uterine abnormalities [15]; however, we were unable to adjust these critical confounders in the present study. Selection bias, which is one of the cardinal drawbacks of retrospective study design, may have effect on our study. In our clinic, HA-rich transfer medium had not been used before October 2013; most subsequent blastocyst transfers were performed using HA-rich transfer medium. For this reason, we have no specific criteria for inclusion and exclusion in the present research. However, it is possible that inadequate embryos were less likely to be chosen in the control group, although there were no specific selection criteria for use of HA-rich transfer medium prior to ET.

In conclusion, our results suggest that the use of HA-rich transfer medium in blastocyst transfer does not appear to have any significant effect on the implantation and pregnancy rates in patients with previous implantation failure. Further large-scale prospective randomized trials are needed to clarify these preliminary results.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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