A randomized clinical trial comparing recombinant hyaluronan/recombinant albumin versus human tubal fluid for cleavage stage embryo transfer in patients with multiple IVF-embryo transfer failure

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**BACKGROUND:** We aimed to examine the efficacy of using an embryo transfer medium enriched with hyaluronan (HA) to improve implantation in a selected group of patients aged <43 years with repeated (≥4) implantation failures after IVF-embryo transfer. **METHODS:** About 101 patients, meeting our selection criteria, were randomly allocated to undergo embryo transfer either using our routine embryo transfer medium without HA (control group) or a HA enriched commercial embryo transfer medium (study group). The primary outcome was clinical pregnancy rate. **RESULTS:** After a similar treatment protocol, the ovarian hormonal response, the mean number of ova retrieved and injected per patient, fertilization and cleavage rates and mean embryo quality were comparable between the study and control groups. Although a similar number of embryos was transferred in both groups (3.1 and 2.9 ± 0.6, mean ± SD), a significantly higher implantation rate (16.3% versus 4.8%, \(P = 0.002\)) and clinical pregnancy rate (35.2% versus 10.0%, \(P = 0.004\)) and delivered or ongoing pregnancy rate (31.3% versus 4.0%, \(P = 0.0005\)) were observed in the study group. When mean implantation rate per patient was calculated, the difference between the study (0.148 ± 0.23) and control (0.04 ± 0.13) group was significant (\(P = 0.003\)). **CONCLUSIONS:** In this selected group of patients after multiple IVF-embryo transfer failures, the use of HA enriched embryo transfer medium is beneficial.

**Keywords:** hyaluronan; implantation; human embryo implantation; recurrent IVF-embryo transfer failure; human tubal fluid

**Introduction**

A major limiting step in the success of the IVF-embryo transfer procedure lies in the implantation. Means to affect it directly are scarce as we lack clinical tools to diagnose and treat the cause of implantation failure. Although several potential biochemical markers of uterine receptivity were investigated (Giudice, 1999), presently, there are none on a clinically available basis. Means to improve implantation are used on an empirical basis. Recently, some evidence has been presented concerning the beneficial effect of hyaluronan (HA), a naturally existing macromolecule abundant in human fluid secretions and extracellular matrix of the reproductive tract, on *in vitro* embryo development and implantation, first in the mouse [Gardner et al., 1999; Gardner and Lane, 2000 (abstract)] then later in human (Schoolcraft et al., 2002 (abstract); Simon et al., 2003; Balaban et al., 2004 (abstract); Balaban and Urman, 2005). Considering the physico-chemical properties of HA, one may hypothesize that it may have a role in assisting the embryo–endometrial interaction during the early phases of implantation. Hyaluronic acid, a linear polysaccharide of alternating \(\alpha\)-glucuronic acid and \(N\)-acetyl-\(\alpha\)-glucosamine residues, is a naturally existing macromolecule related to the glycosaminoglycan family. It is a highly hydrophilic molecule, forming a highly hydrated gel and thereby promoting expansion of the extracellular spaces and facilitating cell migration, metastasis and angiogenesis (Comper and Laurent, 1978; Toole, 1981). HA forms a coating around many types of cells, profoundly affecting their migratory properties and adhesiveness (Laurent and Fraser, 1986). HA is abundant in human fluid secretions and extracellular matrix of the reproductive tract, including the cervical mucus, the cumulus cells, follicular fluid and seminal plasma (Eppif, 1979; Grimek et al., 1984; Binette et al., 1996; Salustri et al., 1999). Also, an HA surface receptor-CD44 is expressed in the human blastocyst (Campbell et al., 1995). Evidence indicates changes in the distribution of HA in the mouse uterus during the peri-implantation period suggesting that it may have an important role in the process of endometrial decidualization and/or embryo implantation (Carson et al., 1987; Brown and Papaioannou, 1992; San Martin et al., 2003).
Recently, a prospective quasi-randomized study reported significantly improved implantation rates using a commercially available HA enriched embryo transfer medium containing recombinant human albumin (EmbryoGlue®, Vitrolife, Englewood, CO, USA) only in a subgroup of patients with tubal factor and recurrent implantation failure (Valojerdí et al., 2006). No significant improvement in the implantation or clinical pregnancy rates was observed when examining the group treated as a whole. Loutradi et al. (2007) reported no improvement in clinical pregnancy rates in a non-selected group of patients using EmbryoGlue® compared to a medium containing a lower concentration of HA and human serum albumin. Encouraged by the findings of an uncontrolled-retrospective study performed in our IVF unit, we performed a prospective randomized study aiming to examine the efficacy of EmbryoGlue® and to evaluate its potential benefit in a selected group of patients who repeatedly failed to achieve pregnancy after IVF-embryo transfer.

Materials and Methods

Study population
Patients aged <43 years, undergoing ICSI at the IVF unit at Assaf Harohef Medical Center during the period of June 2005–November 2006, who suffered from repeated failure of implantation following embryo transfer were included in a prospective randomized study, after Institutional Review Board approval was obtained. There was no financial conflict of interest of the investigators and no sponsorship was obtained from the industry to perform this study. The patients included in our study have failed to achieve an ongoing pregnancy after >4 previous embryo transfers, during which 2–4 embryos were transferred each time, including at least one embryo with optimal cleavage rate and morphology (four cells on day 2 or eight cells on day 3, equal-sized blastomeres and <50% fragmentation). Patients aged >43 years suffering from any systemic disease, having an excessive body mass index of >29 kg/m², uterine malformation, evidence of low ovarian response in previous treatment cycles with less than four oocytes retrieved, elevated baseline (day 3) FSH (>12 IU/l), ultrasonographic evidence of hydrosalpinx or participation in any other clinical study were excluded. All patients meeting the study criteria were included after obtaining their consent. No patient refused to participate in the study. All patients included in the study proceeded to the stage of embryo transfer. Randomization to study or control group was performed on the day of embryo transfer based on a computer originated random number sequence. The study comprised 101 patients, with 51 patients randomized to undergo embryo transfer in a HA enriched commercial embryo transfer medium (EmbryoGlue®, Vitrolife), a bicarbonate buffered embryo transfer medium containing a high concentration of hyaluronic acid (0.5 mg/ml) and a low concentration of recombinant human albumin (2.5 mg/ml) (study group). The control group consisted of 50 patients who underwent embryo transfer using our routine embryo transfer medium [human tubal fluid (HTF) medium with gentamycin (Irvine Scientific, Santa Ana, CA, USA)], enriched with 20% serum substitute supplement (Irvine Scientific, Santa Ana, CA, USA), which contains no HA.

Controlled ovarian stimulation and oocyte retrieval
Ovarian stimulation and oocyte retrieval were performed in all patients according to a routine protocol of mid-luteal pituitary down-regulation, using a daily dose of GnRH-a, (nafarelin acetate, 200 mg three times daily, intranasal spray; or busereline, 400 mg three times daily, intranasal spray; or triptoreline, 0.1 mg/day, s.c. injection) followed by controlled ovarian stimulation in an individually adjusted step-up protocol using daily injection of urinary or recombinant gonadotrophins. Oocytes were retrieved 36–40 h after administration of 5000 IU of HCG (Pregnyl, Organon, Pharmagon), given according to the presence of at least two leading follicles of 18–20 mm. Oocyte pick-up was performed by ultrasound guided transvaginal follicular aspiration. All mature oocytes retrieved underwent a routine ICSI procedure. Indications for IVF-embryo transfer included male factor (66%), tubal factor (requiring ICSI due to previous low fertilization rate) (8%), endometriosis (4%), unexplained infertility (12%) and combination of male and female factors (10%). Fertilization was assessed on the following day, 16–18 h post-sperm injection. If two distinct pronuclei were observed, then fertilization was judged to have occurred.

Embryo transfer, luteal support and pregnancy evaluation
Embryonic cleavage and morphological appearance were assessed 48 or 72 h after ICSI, prior to embryo transfer and before patients’ allocation to the control or the study group. The number of cells represented the cleavage rate and a morphological score was given for each embryo (1 being the best and 4 the worst) according to the degree of fragmentation, granularity and similarity in the size of the blastomeres. The patients allocation to study or control group was performed by the chief embryologist, according to a list of computer-generated random number sequence, just before the embryo transfer procedure. Then, prior to embryo transfer, the embryos were equilibrated for a period of 10 min in the embryo transfer medium, and then loaded into the transfer catheter (Wallace catheter, Marlow technologies, Willoughby, OH, USA). The physician performing the embryo transfer as well as the patient were blinded to the transfer medium. Luteal support was given to all patients, starting on the day of embryo transfer (+1), until serum HCG measurement 14 days following the embryo transfer, using micronized progesterone [Utrogestan, Basins Iscovesco (C.T.S), Paris, France, vaginal tablets, 100 mg three times daily]. If a viable pregnancy was confirmed by ultrasound examination, progesterone support was continued until the 8th week of gestation. Only clinical pregnancies including sonographic demonstration of a gestational sac were counted.

Statistical analysis
Statistical evaluation was performed using Student’s t-test, Chi-squared test or Fisher’s exact test, where appropriate. Difference was considered significant at $P < 0.05$. Assuming a significance level of 0.05 and a power of 0.80, it was calculated that for a minimal difference of 15% between the two groups (from ~10% to 25%), the group size needed was 112 patients in each arm. Although no advance plan was made for an interim analysis, due to the impressive difference in the clinical pregnancy rates between the groups, we examined our data after inclusion of 101 patients, since we felt that in view of the higher success rate of patients in the HA group, it would be unfair to continue the study.

Results
The various clinical parameters of the 101 patients included in the study and control groups are presented in Table 1. It is notable that our selected group of patients was relatively young (mean of 32 years old) and had an average of 5.5 previous unsuccessful embryo transfers. Their response to the controlled ovarian stimulation was satisfactory with more than 10 mature oocytes retrieved and injected per patient. Comparing the study to the control group, no significant differences
were found in clinical parameters, including the mean level of estradiol and progesterone on the day of HCG administration (Table 1). Examining various parameters concerning the outcome of the treatment cycle, including the mean number of ova retrieved per patient, injected, fertilized and cleaved, no statistically significant differences were found between the study and control group. Similar number of embryos was transferred in both groups; in the study group, 30 embryo transfers were on day 2 and 21 on day 3, and in the control, 25 transfers were done on day 2, and 25 on day 3. In both groups, mean embryo quality expressed as the mean number of cells and morphology grade were comparable, both for day 2 or day 3 embryos. However, in the study group, a significantly higher implantation rate \((P = 0.002)\) and clinical pregnancy rate \((P = 0.004)\) were observed, compared with the control group (Table 2). Also, when mean implantation rate \((\pm SD)\) was calculated per patient, the difference between the study \((0.148 \pm 0.23)\) and the control \((0.04 \pm 0.13)\) groups was significant \((P = 0.003)\). In the control group, one twin pregnancy occurred among the five clinical pregnancies, compared to five twins and one triplet out of 18 pregnancies in the study group. Two early spontaneous abortions and one ectopic pregnancy occurred in each group. Although these results were not significantly different, because of the small size of the groups, interpretation of the true significance of these secondary outcomes is limited. Examining the delivered or ongoing pregnancy rates, the difference in favor of the study group increased and reached a statistical significance of \(P = 0.0005\).

### Discussion

Preparation for embryo implantation requires extensive adaptation of the uterine microenvironment involving an orchestrated synchrony of complex interactions between a viable, well-developed embryo and the hormonally primed receptive endometrium (Weitlauf, 1994; Yoshinaga, 1988). Theoretically, implantation can fail due to an inappropriate embryonic function required for implantation, possibly also due to a genetic incompetence and/or inappropriate uterine receptivity. Lacking accurate prospective diagnostic tools that specify the exact problem in the implantation process, the means to improve implantation rates are used on an empirical basis. On the one hand, methods aiming at the improvement of embryo quality include better ovarian stimulation protocols, improved embryo growth media and various co-cultures (Veiga et al., 1999). On the other hand, methods to improve endometrial receptivity include modified ovarian stimulation protocols, improved embryo growth media and various co-cultures (Veiga et al., 1999), administration of leukemia inhibitory factor (Aghajanova, 2004), performance of endometrial biopsies prior to the treatment cycle (Barash et al., 2003; Raziel et al., 2007) and incorporation of various ingredients into the embryo transfer media, such as fibrin glue (Feichtinger et al., 1990; Ben-Rafael et al., 1995), heparanase (not yet reported in human) (Revel et al., 2005) or HA [Gardner et al., 1999; Gardner and Lane 2000 (abstract); Schoolcraft et al., 2002 (abstract); Simon et al., 2003; Balaban et al., 2004 (abstract)]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study group (EmbryoGlue)</th>
<th>Control group (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>33.1 (5.1)</td>
<td>31.7 (5.6)</td>
</tr>
<tr>
<td>Number of previous cycles</td>
<td>5.8 (2.6)</td>
<td>5.2 (1.4)</td>
</tr>
<tr>
<td>hCG-day estradiol level (pg/ml)</td>
<td>2252 (1309)</td>
<td>2187 (1327)</td>
</tr>
<tr>
<td>hCG-day progesterone level (ng/ml)</td>
<td>1.0 (0.61)</td>
<td>1.0 (0.57)</td>
</tr>
<tr>
<td>Number of ova retrieved per patient</td>
<td>12.0 (7.3)</td>
<td>13.3 (6.2)</td>
</tr>
<tr>
<td>Number of mature (2PN) ova injected per patient</td>
<td>10.3 (6.6)</td>
<td>10.6 (5.0)</td>
</tr>
<tr>
<td>Number of mature (2PN) ova fertilized per patient</td>
<td>7.0 (4.2)</td>
<td>7.0 (3.6)</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>70.0 (16.1)</td>
<td>68.0 (19.0)</td>
</tr>
<tr>
<td>Number of embryos cleaved</td>
<td>6.9 (3.9)</td>
<td>6.8 (4.0)</td>
</tr>
<tr>
<td>Number of embryos transferred</td>
<td>3.1 (0.73)</td>
<td>2.9 (0.65)</td>
</tr>
<tr>
<td>Mean embryo quality</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>3.77 (1.10)</td>
<td>3.88 (1.03)</td>
</tr>
<tr>
<td>Day 3</td>
<td>7.81 (0.62)</td>
<td>7.59 (1.02)</td>
</tr>
<tr>
<td>Morphology grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 2</td>
<td>1.89 (0.59)</td>
<td>1.88 (0.53)</td>
</tr>
<tr>
<td>Day 3</td>
<td>1.57 (0.54)</td>
<td>1.70 (0.56)</td>
</tr>
</tbody>
</table>
HA was present in the transfer medium, it is possible that the difference in HA concentration was not great enough to cause a significant rise in implantation rate, especially in a non-selected group of patients.

To examine the potential benefit of using embryo transfer medium enriched with HA, we conducted the study in a highly selected group of patients who repeatedly (>4 treatments, six times on average) failed to achieve pregnancy after IVF-embryo transfer. Our randomization was performed by a computer generated random number sequence. Characteristically, these patients were good responders with an average of 10 mature oocytes retrieved, satisfactory fertilization rate of 70% leading to the development of good quality embryos reaching four cells on day 2 with grade 2 morphology score. However, their implantation (6.9%) and clinical pregnancy rates (9.8%) were low, as shown in the results of the control group. Our findings corroborate the results reported by Valojerdi et al. (2006) regarding the beneficial effects of EmbryoGlue® in patients with repeated IVF-embryo transfer failures. Our inclusion criteria were stricter as we included only patients with at least four previous unsuccessful transfers. The significant improvement observed in the embryo implantation rate and the prospect to achieve a clinical pregnancy in the study group indicates that in this selected group of patients, the use of a commercially available embryo transfer medium enriched with HA was beneficial.

It is not clear whether the beneficial effect of HA enriched transfer medium is related to improvement in the uterine receptivity or enhancement of the embryos implantation potential. As pointed out by Simon et al. (2004) HA, by virtue of its physical properties, produces a viscous solution that might enhance the embryo transfer process and prohibit the expulsion of embryos from the uterine cavity after transfer. In addition, it has been suggested that the use of HA in transfer media can facilitate the diffusion and integration of the embryos in the viscous solution characterizing the intrauterine secreted fluid (Gardner et al., 1999). Moreover, as mentioned by Correa-Perez (2004), it has been shown that HA is involved in cell–cell and cell–matrix adhesion and early embryo development (Salustri et al., 1999; Okada et al., 2001; Salomonsen et al., 2001). One must note that the patients in our study could still be heterogeneous in their etiology for implantation failure, as no direct tests are available to exactly diagnose the specific problem. Also the relatively small size of groups included in the study warrant further research. In conclusion, our findings definitely should encourage the performance of further prospective randomized studies aiming to validate the efficacy of HA addition to the embryo transfer medium to improve human embryo implantation following IVF-embryo transfer in a selected group of patients suffering from multiple implantation failures.

References

Gardner DK, Rodriguez-Martinez H, Lane M. Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. Hum Reprod 1999;14:2575–2580.

Table 2: ICSI outcome in the study and control groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study group (EmbryoGlue) n = 51</th>
<th>Control group n = 50</th>
<th>P-value, relative risk, confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Implantation rate (%)</td>
<td>16.3 (26/159)</td>
<td>4.8 (7/146)</td>
<td>P = 0.002, RR = 3.39, CI: 4.85–18.27</td>
</tr>
<tr>
<td>Clinical pregnancy rate (%)</td>
<td>35.2 (18/51)</td>
<td>10.0 (5/50)</td>
<td>P = 0.0045, RR = 3.52, CI: 9.76–40.82</td>
</tr>
<tr>
<td>Multiple pregnancy rate (%)</td>
<td>33.3 (6/18)</td>
<td>20.0 (1/5)</td>
<td>P = 1.0, RR = 1.6, CI: −27.94 to 54.61</td>
</tr>
<tr>
<td>Early spontaneous abortion rate (%)</td>
<td>11.1 (2/18)</td>
<td>40.0 (2/5)</td>
<td>P = 0.038, RR = 0.27, CI: −74.22 to 16.44</td>
</tr>
<tr>
<td>Ectopic pregnancy</td>
<td>1.96 (1/51)</td>
<td>2.0 (1/50)</td>
<td>P = 1.0, RR = 0.98, CI: −5.47 to 5.40</td>
</tr>
<tr>
<td>Delivered or ongoing pregnancy rate (%)</td>
<td>31.3 (16/51)</td>
<td>4.0 (2/50)</td>
<td>P = 0.0005, RR = 7.82, CI: 13.53–41.22</td>
</tr>
</tbody>
</table>

CI = 95% confidence interval.


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