Efficacy of a human embryo transfer medium: a prospective, randomized clinical trial study

Mojtaba Rezazadeh Valojerdi,^{1,2,3} Lila Karimian,¹ Poopak Eftekhari Yazdi,¹
Mohammad Ali Sadighi Gilani,¹ Tahereh Madani,¹ and Ahmad Reza Baghestani¹

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Purpose: The aim of this prospective, randomized trial was to evaluate the efficacy of Embryo-Glue® as a human embryo transfer medium in IVF/ICSI cycles.

Method: A total of 815 nonselected patients undergoing IVF/ICSI treatment between September 2003 and February 2004 were randomly allocated into the test (417 patients) and the control (398 patients) groups. In both groups, embryos were cultured in G-1™ver 3, supplemented with 10% recombinant human albumin. On the day of embryo transfer (day 3), the best or good quality embryos were selected for intrauterine transfer. In the test group, the selected embryos were treated with EmbryoGlue® prior to the transfer, whereas in the control group they were transferred without any treatment.

Results: The patients' characteristics such as age and the number of ART cycles and also the number of patients in each indication of infertility and the number of embryos selected for transfer were all similar between the two groups. In the test group, the clinical pregnancy rate in the tubal factors and the implantation rate in the tubal factors and recurrent implantation failures increased significantly compared with those in the control group. In the test group, life birth and the triplet delivery rates increased significantly compared with those in the control group.

Conclusion: EmbryoGlue® is a useful embryo transfer medium, and at least in some infertile patients it can improve clinical implantation and ongoing pregnancy rates.

KEY WORDS: Clinical pregnancy rate; Embryoglu®; implantation rate; recurrent implantation failures; Tubal factors.

INTRUDUCTION

Today, commercial media are widely used for oocyte retrieval, embryo culture, sperm preparation, and embryo transfer. These media are primarily based on the role of ions, amino acids, and carbohydrate. However, in a number of new investigations, a

few authors have focused on the role of different macromolecules in embryo culture media. One of these macromolecules is albumin, which has been traditionally used as the main macromolecule in most culture media used for in vitro growth of human embryos (1). Albumin, highly abundant in the female reproduction tract (2), serves as a source of energy and as a reservoir for the release of hormones, vitamins, and metals. In addition, the presence of albumin in culture media not only provides the useful physical properties of lubrication and viscosity but it also promotes ease of handling the embryos and prevents its adherence to the culture dish. The sources of albumin for culture

¹ Infertility Research Center, Royan institute, Tehran, Iran.

² Department of Anatomy, School of Medical Sciences, Tarbiat Modarres, University, Tehran, Iran.

³ To whom correspondence should be addressed at Department of Embryology, Royan Institute, Simin St., Asef Cross, P.O. Box: 19395-4644, Zafaranieh, Tehran, Iran; e-mail: info@royaninstitute.org.

media are the patient's own serum, fetal cord serum, commercially pooled human serum albumin (HSA) and lately, recombinant human albumin (rHA).

Another macromolecule that has been suggested recently as a promising factor in culture media is hyaluronan (hyaluronic acid) (3–5).

Hyaluronan is a linear polysaccharide of alternating D-glucuronic acid and N-acetyl-D-glucosamine residues. Hyaluronan is a major glycosaminoglycans present in the cervical mucus, the cumulus, follicular fluid, and seminal plasma (6–8). This macromolecule, also present in the oviduct and uterine fluids, increases up to the time of implantation (9). Gardner *et al.* (3,10) have demonstrated that mouse embryos can successfully grow and implant when cultured and transferred in a medium containing hyaluronan extracted from the rooster comb or as a recombinant substance. In addition, Hyaluronan has been shown to have an implantation-promoting effect by Schoolcraft *et al.* (11), who demonstrated that EmbryoGlue® significantly increased the implantation rate compared to a blastocyst culture medium (G-2™ver 3, Vitrolife, Sweden). EmbryoGlue® is a modification of G-2™ver 3, with higher concentration of hyaluronan and HSA replaced with rHA as the only differences between the two media. Stojkovic *et al.* (5) have demonstrated that a high concentration of hyaluronan increases the viscosity of the culture medium and improves in vitro development and the number of cells of bovine embryos. They have also observed higher survival rates after freezing of embryos cultured with hyaluronan supplementation.

In a recent study, Simon *et al.* (1) have demonstrated that hyaluronan can successfully replace albumin as a macromolecule in a human embryo transfer medium and result in high pregnancy and implantation rates. Taking these hypotheses and the role of hyaluronan in culture media into account, the Vitrolife Company generates EmbryoGlue® as a human embryo transfer medium. This medium contains a high concentration of hyaluronan (0.5 mg/mL) and a low concentration of rHA (2.5 mg/mL). Accordingly, the present prospective, randomized clinical trial study was undertaken to evaluate the efficacy of EmbryoGlue® as a human embryo transfer medium and to compare the clinical outcomes versus those of G-1™ver 3, which is supplemented with 10 mg/mL of rHA and contains a lower concentration of hyaluronan (0.125 mg/mL) compared with EmbryoGlue®.

Other differences between EmbryoGlue® and G-1™ver 3 are presence of EDTA, nonessential

and essential amino acids. According to Gardner *et al.* (12), EDTA stimulates cleavage stage embryo development but inhibits blastocyst development and differentiation. EDTA is therefore present in G-1™ver 3 but not in EmbryoGlue® since it is not considered beneficial to introduce EDTA into the uterus. Furthermore, Lane *et al.* (13,14) have studied the roles of amino acids in embryo development and conclude that cleavage stage embryos benefit from nonessential amino acids but not from essential amino acids, which may even impair development at that stage. On the other hand, essential amino acids as well as nonessential amino acids are very important for the development of the blastocyst. For the above reasons, G-1™ver3 contains nonessential amino acids while EmbryoGlue®, which is based on a blastocyst culture medium, contains both nonessential and essential amino acids.

MATERIALS AND METHODS

Patients

A total of 815 patients participated in this study, which was carried out between September 2003 and February 2004 at the assisted conception unit, Royan Institute, Tehran, Iran. The patients underwent ICSI/IVF treatment and their embryos were selected for intrauterine transfer by the chief technician in lab without any interference of the embryologists. The embryo transfer procedure was then performed every other day with EmbryoGlue® ($n = 417$ patients as a test group) or G-1™ver3 ($n = 398$ patients as a control group).

Human Spermatozoa Preparation

The female's partner produced semen either by masturbation (in 727 patients) or by testicular biopsy and epididymal aspiration (In 88 patients). In the case of the ICSI procedure, spermatozoa were prepared for injection by the standard swim-up technique. Whereas in the IVF procedure, spermatozoa were prepared for insemination by differential centrifugation using PureSperm™ (Nidacon, Sweden). Final sperm suspension was made in Ham's-F10 culture medium, supplemented with 10% Albuminar-5 (containing 5% human serum albumin, Blood Research Center, Iran). The prepared spermatozoa were incubated under a gaseous phase of 6% CO₂ in air at 37°C until use.

Ovarian Stimulation and Oocyte Retrieval

Ovarian stimulation was performed following downregulation as previously described (15). In brief, suppression of pituitary gonadotropin secretion with GnRH agonist (GnRH-a) buserelin acetate (superfact, Hoechst AG, Germany) at the dose of 500 μ g/day by subcutaneous injection or 800 μ g/day by nasal spray was commenced in the mid-luteal phase of the preceding ovarian cycle (day 21). Once ovarian suppression was confirmed (serum oestradiol $\leq$ 50 pg, LH $\leq$ 5 IU/mL), ovarian stimulation was initiated using a subcutaneous injection of 150 IU/day purified human menopausal gonadotropin (hMG; pergonal 500, Serono, Italy). The dose was increased in tandem with ovarian follicular development, monitored by serial vaginal ultrasonography when at least three follicles reached 18 mm in diameter. GnRH-agonist and hMG were discontinued, and 10000 IU of human chorionic gonadotropin (hCG; Organon; Netherlands) was administered. Oocyte retrieval was performed by ultrasound-guided follicle aspiration, 36–38 h after hCG administration. Standard IVF (In 158 patients) or ICSI procedure (in 657 patients) was used to achieve oocyte fertilization.

Culture Procedure

Over the study period, there were no changes in the laboratory procedures and the culture media utilized. In the case of IVF, the oocyte–cumulus masses were collected in a drop of Ham's F-10 medium, supplemented with 10% Albuminar-5 and washed in the G-1TMver 3 (Vitrolife, Sweden) supplemented with 10% recombinant serum albumin (rHA, Vitrolife, Sweden). Next, they were transferred into a 20 μ L fresh G-1TMver 3 medium, held under mineral oil in the culture dish. The oocytes were then inseminated with 50,000/mL spermatozoa, incubated overnight in an atmosphere of 6% CO₂ in air at 37°C.

In the case of ICSI, the oocytes were injected in the HEPES Ham's F-10 medium, supplemented with 10% Albuminar-5 and washed in the G-1TMver 3 supplemented with 10% rHA. After that, they were transferred into a 5- μ L fresh G-1TMver 3 medium, held under mineral oil in the culture dish. The injected oocytes were then incubated with the same manner.

After 12–18 h of cultivation, the oocytes were assessed for fertilization in both the IVF and ICSI cases. The normal fertilized oocytes were then transferred into a fresh drop of the G-1TMver 3,

supplemented with 10% rHA, and were incubated for another 24 h.

After 36–40 h of cultivation, cleaved embryos were classified according to the following quality criteria: excellent quality ($\geq$ 4 cells and $<$ 10% fragmentation), good quality ($\geq$ 4 cells and 10–20% fragmentation) or poor quality ($<$ 4 cells and $>$ 20% fragmentation).

Embryo Transfer Procedure

After 40–44 h of cultivation, the best or good quality of embryos were selected for intrauterine transfer (maximum 3–4 embryos in each embryo transfer cycle) by our chief technician without any interference of the embryologists. In the control group, the selected embryos were transferred into a 50 μ L fresh culture medium, whereas in the test group, they were transferred into 50 μ L EmbryoGlue[®] (Vitrolife, Sweden), preincubated 4–18 h in an environment of 6% CO₂ in air at 37°C. After 20 min, all embryo transfers were performed using a Labotect catheter (Labotect, Germany) while the physician was blinded to the transfer medium.

The luteal phase was supported by intravaginal progesterone (Cyclogest 400, Coxph, U.K.) in two divided doses for a total amount of 800 mg/day starting 1 day after oocyte retrieval.

The patients were tested for serum β -hCG assay 15 days after embryo transfer. If the pregnancy test was positive, the patients were followed with serial ultrasounds to determine fetal viability. Clinical pregnancy was defined as the presence of a gestational sac on transvaginal ultrasound. When pregnancy occurred, luteal support was continued until 12 weeks' gestation.

Statistical Analysis

Results were analyzed using student's *t*-test and chi-square test. A *p*-value of $<$ 0.05 was considered significant.

RESULTS

The patients' characteristics and the clinical outcomes of embryo transfer in the test and control groups are summarized in Table I. The mean of the females' ages, the number of ART cycles, the number of patients in each indication of infertility, the number of patients with recurrent implantation

Table I. Comparison of Patients' Characteristics and Clinical Outcome of Embryo Transfer Between Test (EmbryoGlue®) and Control (G-1™ver 3) Groups

	Test group	Control group
IVF/ICSI cycles	417 (51.2)	398 (48.8)
Females' age (Mean $\pm$ SD)	31.4 $\pm$ 5.4	31.4 $\pm$ 5.7
Male factors	181 (43.5)	165 (41.5)
Tubal factors	50 (12.0)	48 (12.1)
Ovarian disorders	71 (17.0)	67 (16.8)
Idiopathics	30 (7.2)	32 (8.0)
Endometriosis	13 (3.1)	4 (1.0)
Multiple factors	67 (16.1)	80 (20.1)
Uterine factors	5 (1.2)	2 (0.5)
Number of recurrent Implantation failures (Mean $\pm$ SD)	1.5 $\pm$ 1	1.6 $\pm$ 1.1
Number of oocytes retrieval (Mean $\pm$ SD)	7.3 $\pm$ 4.9	7.1 $\pm$ 4.4
Number of fertilized oocytes (Mean $\pm$ SD)	5.2 $\pm$ 4	4.8 $\pm$ 3.7
Number of embryos transferred/cycles (Mean $\pm$ SD)	2.9 $\pm$ 1.1	2.9 $\pm$ 1.1
Clinical pregnancy rate/transfer cycle	130/417 (31.2)	116/398 (29.1)
Implantation rate/embryo transfer	186/1230 (15)	151/1166 (13)
Multiple pregnancy/pregnant patient	40/130 (30.8)	30/116 (25.9)

Note. Values in parentheses are percentage; no statistical differences were found between the two groups.

failure (≥ 2 cycles), the number of oocytes retrieval, the number of fertilized oocytes and the number of embryos selected for transfer were all similar between the two groups. In the test groups, the rates of pregnancy/transfer cycle, implantation/embryo transfer and multiple pregnancy/pregnant patient were numerically higher than those in the control group. The differences, however, were not significant (31.2, 15, 30.8 versus 29.1, 13 and 25.9%, respectively).

The clinical outcomes of embryo transfer between the test and control groups in the patients with recurrent implantation failures (≥ 2 cycles) and in the patients with different indications of infertility are summarized in Table II. In the test group, the clinical pregnancy rate increased significantly in the patients with the tubal factor (38 versus 18.8%, $p < 0.05$), whereas the implantation rate increased significantly in the patients with the tubal factor (20.5 versus 8.9%, $p < 0.05$) and in the patients with recurrent implantation failures (16 versus 11%, $p < 0.05$). The difference in multiple pregnancy rate not significant (26.3 versus 22.7% for the tubal factors and 32.6 versus 23.7% for recurrent implanta-

tion failures). In a subgroup analysis within the test group, some infertile patients such as those suffering from male factors, ovarian disorders, idiopathics, endometriosis, uterine factors and multiple factors no significant differences in pregnancy rate or implantation rate could be found. In the test group, the rates of life birth/embryonic sac and triplet deliveries increased significantly compared with those in the control group (74.5 versus 62.5% for the former and 9.5 versus 1.4% for the latter, $p < 0.05$) (Table III).

DISCUSSION

In the present study, human embryos were cultured for 44–48 h in G-1™ver 3 enriched with 10 mg/mL rHA followed by a random transfer in either the same medium or in EmbryoGlue® transfer medium. A relatively high and comparable clinical pregnancy rate was achieved in both groups (31.2 and 29.1% for the EmbryoGlue® and G-1™ver 3 groups, respectively). Although the number of embryos selected for transfer was similar between the two groups, the multiple pregnancy rate for the EmbryoGlue® reached 30.8%, whereas that of the G-1™ver 3 group was 25.9%. This was due to the implantation rate, which was somewhat higher for the EmbryoGlue® group compared with the G-1™ver 3 group (15 and 13%, respectively). These results were in agreement with those achieved by Simon *et al.*'s study (1), in which the sole macromolecules in the transfer medium for the test and control groups were hyaluronan and HSA, respectively, and the patients were younger than 35 years and had no recurrent implantation failures. However, they failed to demonstrate any significant differences between the test and control groups.

The somewhat higher clinical and implantation rates in our test group may be due to the presence of hyaluronan in the EmbryoGlue® transfer medium as shown by Schoolcraft *et al.* (11) and Balaban *et al.* (16). The precise mechanism of action of hyaluronan in the process of implantation still is not clear. However, these are several possible means by which hyaluronan could facilitate implantation; (i) hyaluronan has been shown to increase cell–cell adhesion and cell–matrix adhesion (17), and so it may function during the initial stages of apposition and attachment of the blastocyst and endometrium (3); (ii) hyaluronan has several main functions which include

Table II. Comparison of Clinical Outcome of Embryo Transfer Between Test (EmbryoGlue®) and Control (G-1™ver 3) Groups in Patients With Recurrent Implantation Failures and Different Indications of Infertility

	Test group			Control group		
	PR	IR	MP	PR	IR	MP
Recurrent implantation failures (≥ 2 cycles)	43/128 (33.6)	65/407 (16)*	14/43 (32.6)	38/147 (25.9)	48/436 (11)	9/38 (23.7)
Male factors	60/181 (33.1)	85/556 (15.3)	12/60 (20)	51/165 (30.9)	69/494 (14)	7/51 (13.7)
Tubal factors	19/50 (38)*	31/151 (20.5)*	5/19 (26.3)	9/48 (18.8)	12/135 (8.9)	2/9 (22.7)
Ovarian disorders	18/71 (25.4)	25/218 (11.5)	4/18 (22.2)	19/67 (28.4)	25/197 (12.7)	4/19 (21/1)
Idiopathics	12/30 (40)	19/82 (23.2)	4/12 (33)	14/32 (43.8)	15/97 (15.5)	1/14 (7)
Endometriosis	4/13 (30.8)	4/32 (12.5)	0/4 (0)	0/4 (0)	0/14 (0)	0/0 (0)
Multiple factors	12/67 (17.9)	17/176 (9.7)	3/12 (25)	23/80 (28.8)	29/224 (12.9)	5/23 (21)
Uterine factors	5/5 (100)	5/15 (33)	0/5 (0)	1/2 (50)	1/5 (20)	0/1 (0)

Note. Values in parentheses are percentage; PR: Pregnancy rate; IR: Implantation rate; MP: Multiple pregnancy rate.

* $p < 0.05$.

cell–cell adhesion, cell–matrix adhesion, regulation of protein secretion, gene expression cell proliferation and differentiation (18,19); therefore, it may function during the initial stages of apposition and attachment of the blastocysts and endometrium (3); (iii) hyaluronan can promote angiogenesis by both its degradation products (20) and by interaction with epidermal growth factor (EGF) (21). The latter point is of particular interest given the stimulatory effect of EGF on implantation in the mouse (22); (iv) hyaluronan, by its physical property, produces a viscous solution that might enhance the embryo transfer process and prohibit the expulsion of embryos from the uterine cavity (3). Gardner *et al.* (3) also explain that as the uterine fluid is a viscous solution, the transfer of a relatively aqueous solution such as culture medium with albumin to the uterine lumen will result in the slow dispersal of the medium and embryo with the luminal contents. In contrast, the transfer of an embryo in a hyaluronan solution will facilitate dispersal of the embryo into the luminal environment; and (v) hyaluronan also confers some degree of viral protection and anti-immunogenic properties which inhibit the rejection of embryos from the uterine cavity (23).

One of the interesting findings in our investigation was the observation of clinical outcome in the different indications of infertility. Accordingly, in the EmbryoGlue® group, the clinical pregnancy rate increased in the patients with tubal factors (38 versus 18.8%, $p < 0.05$) and for the implantation rates in the both tubal factors (20.5 versus 8.9%, $p < 0.05$) and recurrent implantation failures (16 versus 11%, $p < 0.05$) compared with those in the G-1™ver 3 group. Such results have been previously reported elsewhere by Schoolcraft *et al.* (11) and Balaban *et al.* (16), who used EmbryoGlue® and reported better

implantation rates in patients with recurrent implantation failures. In contrast to these findings, our investigation also indicated that in the EmbryoGlue® group, some figures of the clinical outcome in the patients with ovarian disorders, idiopathics and multiple factors were lower than those in the G-1™ver 3 group. This contradiction may be related either to the high viscosity of the uterine secretion in the latter groups of infertile patients, in which the uterine secretion can not be dispersed even by hyaluronan of the EmbryoGlue® or to unknown reasons.

Another interesting finding in our investigation was the follow-up of the pregnant patients. To date, 113 patients in the EmbryoGlue® and 109 patients in the G-1™ver 3 group have been followed up successfully. In the EmbryoGlue® group, 84 have given birth (120 healthy newborns) for a take-home baby rate of 74.5%. This rate has been higher than that of the G-1™ver 3 group, in which 71 patients have given birth (90 healthy newborns) for a take-home baby rate of 62.5%. The higher take-home baby rate in the EmbryoGlue® group is not only because of a higher delivery rate, but also due to the higher multiple deliveries. In this group, the singleton and twin deliveries were similar to those in the G-1™ver

Table III. Comparison of Delivery and Life Birth Rates Between Test (EmbryoGlue®) and Control (G-1™ver 3) Groups

	Test group	Control group
Delivery rate	84/113 (74.3)	71/109 (65.1)
Life birth/embryonic sac	120/161 (74.5)*	90/144 (62.5)
Singelton	56/84 (67)	53/71 (75)
Twin	20/84 (23.8)	17/71 (23.9)
Triplet	8/84 (9.5)*	1/71 (1.4)

* $p < 0.05$.

3 group, whereas triplet deliveries noticeably increased compared with those in the G-1TMver 3 group (9.5 versus 1.4%, $p < 0.05$).

CONCLUSIONS

The results from the present study demonstrate that (i) EmbryoGlue[®] is a useful embryo transfer medium, and at least in some infertile patients suffering from recurrent implantation failures and tubal factors can improve the clinical outcome. However, to achieve better results, some modification such as treating embryos with EmbryoGlue[®] for more than 20 min, shifting the embryo transfer program from day 3 to day 5 of embryo development and changing the concentration of rHA and hyaluronan in EmbryoGlue[®] may still be required and (ii) EmbryoGlue[®] generally increases the chance of multiple pregnancy; therefore, it is necessary to reduce the number of selected embryos for intrauterine transfer while this medium is used in the IVF program.

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