Effect of hyaluronan-enriched transfer medium on implantation and pregnancy rates after day 3 and day 5 embryo transfers: a prospective randomized study

Bulent Urman, M.D., Kayhan Yakin, M.D., Baris Ata, M.D., Aycan Isiklar, M.Sc., and Basak Balaban, B.Sc.

Assisted Reproduction Unit, American Hospital of Istanbul, Istanbul, Turkey

Objective: To analyze whether the use of a hyaluronan-enriched transfer medium (HETM) increases rates of implantation (IRs) and clinical pregnancy (CPRs), compared with the use of a conventional transfer medium after day 3 and day 5 embryo transfers.
Design: Prospective randomized controlled trial.
Setting: An assisted reproduction program in a private tertiary-care hospital in Turkey.
Patient(s): A total of 1,282 consecutive fresh embryo transfer cycles (825 day 3 and 457 day 5) were randomly allocated into two groups. In 639 women, ET was effected with HETM, and in 643, it was effected with a conventional embryo transfer medium.
Intervention(s): Embryo transfer using HETM or conventional embryo transfer medium.
Main Outcome Measure(s): Clinical pregnancy rates and IRs were compared with regard to day of embryo transfer, women's age, quality of the transferred embryos, and presence of previous implantation failures.
Result(s): Overall CPRs and IRs significantly increased with the use of HETM (CPR: 54.6% vs. 48.5%, odds ratio: 1.28, 95% confidence interval: 1.03–1.59; IR: 32% vs. 25%, odds ratio: 1.43, 95% confidence interval: 1.23–1.66, for HETM and control groups, respectively). The number needed to treat (NNT) for one additional pregnancy with routine use of HETM was 17. The beneficial effect was more prominent in women who were >35 years of age (NNT = 7), in women who had previous failed cycles (NNT = 7), and in women who had poor-quality embryos (NNT = 8).
Conclusion(s): The enrichment of transfer medium with hyaluronan increases CPRs and IRs, both for day 3 and day 5 embryo transfers. The beneficial effect was most evident in women who were >35 years of age, in women who had only poor-quality embryos available for transfer, and in women who had previous implantation failures. (Fertil Steril® 2008;90:604–12. ©2008 by American Society for Reproductive Medicine.)

Key Words: Hyaluronan, embryo transfer, pregnancy rate, implantation rate

Embryo culture media have evolved over the decades from simple salt solutions to more complex media that modulate the differing needs of the embryo throughout the in vitro culture period. The basic principle in the evolution of culture media is to mimic in vivo conditions and create an environment similar to that of the female reproductive tract. Sequential media that are used today mirror to a certain degree the changing environment within the female reproductive tract (1, 2).

Transfer of embryos generated through IVF–intracytoplasmic sperm injection and grown in in vitro culture conditions results in a successful pregnancy in 15%–60% of cases, depending on their stage of development (3, 4). Traditionally, the medium that is used for embryo transfer has been similar or identical to the solution that is used for growing the embryos. Although extensive research has been performed on in vitro culture media, and various sequential media have been developed that mimic the in vivo environment, the role and composition of the embryo transfer medium were not studied extensively as well. The media used for embryo culture and transfer are almost pure aqueous solutions supplemented with protein, usually at 5% to 10%. This protein is usually in the form of serum albumin or whole serum. However, such protein-supplemented media are not similar to the viscous fluids of the female reproductive tract. In an attempt to increase the viscosity of the transfer medium, synthetic macromolecules such as polyvinyl alcohol or 1% placental collagen have been suggested (5, 6). However, there was no increase in implantation rates as a result of their use.

Although albumin is the most abundant macromolecule in the female reproductive tract, glycosaminoglycans, particularly hyaluronan, also are found at high concentrations (7–10). In a study on mouse embryo development and transfer, Gardner et al. (11) showed that hyaluronan could substitute for serum albumin in culture media. Moreover, it was

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Reprint requests: Bulent Urman, M.D., Assisted Reproduction Unit, American Hospital of Istanbul, Guzelbahce Sokak No. 20, Nisantasi, Istanbul, Turkey 34365 (FAX: 90-212-3112339; E-mail: burman@superonline.com).

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determined that the pre-equilibration of mouse embryos with 0.5 mg/mL of hyaluronan before transfer resulted in a significant increase in subsequent implantation rates. More recently, culture media, supplemented with macromolecules such as recombinant human albumin and hyaluronan, was reported to improve embryo viability after cryopreservation and thawing and to increase cryotolerance (12). In accordance with these findings, we recently showed improved implantation and clinical pregnancy rates for day 3 and day 5 transfers, as well as frozen-thawed embryo transfers, with this recently introduced hyaluronan-enriched transfer medium (HETM, EmbryoGlue; Vitrolife, Kungsbacka, Sweden) (13). The differences compared with commonly used culture media for embryo transfer were the fourfold increase in the concentration of hyaluronan (0.5 mg/mL vs. 0.125 mg/mL), the fourfold decrease in the concentration of recombinant human albumin (2.5 mg/mL vs. 10 mg/mL), and the absence of ethylenediaminetetraacetic acid (EmbryoGlue and G2, GIII series, Vitrolife).

Recently, two studies were published that had contradictory results regarding embryo transfer outcome with this transfer medium (14, 15). Although Valojerdi et al. (14) reported increased pregnancy rates in tubal-factor infertility in a prospective randomized trial when using the HETM, Loutradi et al. (15) reported no benefit of HETM in a retrospective study.

The aim of the current study was to answer the following two questions in a prospective randomized manner: [1] does this new transfer medium have a significant impact, compared with conventional embryo transfer medium, on implantation and pregnancy rates after cleavage stage and blastocyst transfers, and if so, [2] is there a specific group of patients who would benefit from its use?

MATERIALS AND METHODS

A total of 1,282 fresh IVF–intracytoplasmic sperm injection cycles in couples undergoing assisted reproduction treatment with their own gametes, who were eligible for embryo transfer, was randomized to embryo transfer that used either HETM or conventional embryo transfer medium. The study was conducted between June 2006 and June 2007. The last patient was recruited in February 2007. All treatment cycles that reached embryo transfer were considered eligible for inclusion in the study. Patients were randomized according to a previously prepared computer-generated randomization list. Randomization was stratified for day 3 and day 5 transfers. The allocation was disclosed by the nurse coordinator to the chief embryologist who was loading the catheter, after the opening of consecutively numbered sealed opaque envelopes on the morning of the embryo transfer. Each patient was included in the present study with one cycle. The clinician performing the transfer and the patient undergoing embryo transfer were blinded for the allocation. Approval of the institutional review board and informed consent from each patient were obtained.

Stimulation Protocols, Oocyte Retrieval, and Embryo Transfer

Controlled ovarian hyperstimulation was undertaken either with a long-acting GnRH analogue or with an antagonist protocol combined with hMG (Menogon; Ferring, Kiel, Germany) or with recombinant FSH (Gonal-F; Serono, Bari, Italy). In the long protocol, hCG (5,000–10,000 IU) was administered when the leading follicle reached 20 mm in mean diameter, accompanied by two or more follicles of >16 mm. In the antagonist protocol, 250 μg/d of antagonist (Cetrotide, Serono) was administered from the 6th day of stimulation and was continued until the day of hCG. Human chorionic gonadotropin was administered when the lead follicle was ≥ 18 mm in mean diameter. Stimulation protocols and the indications for hCG injection or cycle cancellation did not change throughout the study period.

Oocyte retrieval was undertaken 36 hours after the administration of hCG. Fertilization was achieved by IVF in couples with tubal factor and was achieved by intracytoplasmic sperm injection in couples with any other indications for infertility. Embryo transfer was performed on day 3 or day 5. Transfer was performed under ultrasound guidance with the Wallace (Sims Portex Ltd., Hythe, Kent, United Kingdom) embryo transfer catheter. Cleavage-stage embryos were graded as follows: grade 1, no fragmentation, with equal-sized homogenous blastomeres; grade 2, $<20\%$ fragmentation, with equal-sized homogenous blastomeres; grade 3, 20%–50% fragmentation, with unequal-sized blastomeres; and grade 4, $>50\%$ fragmentation, with unequal-sized blastomeres. Blastocysts were graded as follows: BG1, early cavitation, resulting in an eccentric and then expanded cavity, lined by a distinct inner cell mass region and trophectoderm layer; BG2, delayed initial cavitation exhibiting a transitional phase between early cavitation and expansion; and BG3, blastocysts with several degenerative foci in the inner cell mass region. In BG3, cells appear dark and necrotic, according to Dokras et al. (16).

Embryo Culture

We used GIII series (Vitrolife) culture media and Ovoil (Vitrolife) for in vitro embryo culture throughout the study period. All GIII series culture media besides G-RINSE (Vitrolife) were supplemented with human serum albumin (5 mg/mL). Human serum albumin (HSA solution, 0.5 mL, Vitrolife) was added to 9.5 mL of the solution. After supplementation with HSA, media were stored for a maximum of 7 days at a temperature of 2°C to 8°C. Culture dishes with the media were incubated at 37°C and 6% CO_2, for at least 4 hours before usage. The medium used for handling and manipulating oocytes and embryos (G-MOPS, Vitrolife) was kept in ambient room atmosphere.

Before oocyte retrieval, the needle lumen, the tubing, and the patient's cervix was rinsed with G-RINSE that had been pre-equilibrated at 37°C and 6% CO_2. We used G-MOPS for collecting aspirates and follicle flushing. Collected

oocytes were placed in four-well dishes (Nunclon, Roskilde, Denmark) containing 0.7 mL of G-MOPS, supplemented with HSA, and 0.3 mL of Ovoil, both equilibrated at 37°C.

The culture dishes for this procedure were placed on the heated stage of the laminar air flow device, with the lid on for ≥15 minutes and ≤60 minutes before and during oocyte collection. The oocytes were washed in prewarmed, fresh G-MOPS + HSA and were transferred to a pre-equilibrated (37°C and 6% CO_2) four-well dish containing supplemented G-FERT (Vitrolife) culture media that was used for preparation and handling of gametes and for IVF. Cumulus cell removal was effected 2–3 hours after oocyte retrieval by using hyaluronidase (Hyase-10X, Vitrolife) at a concentration of 10 IU/mL. Oocytes were placed one by one in 10 μL of supplemented G1 medium (Vitrolife) covered with oil that had been pre-equilibrated at 37°C and 6% CO_2. We used G1 media for culture of embryos from pronuclear stage to day 3, and then we used G2 medium. Fertilization was checked 16 to 18 hours after insemination, and each fertilized oocyte was placed in a fresh culture dish containing 10 μL of supplemented G1 medium.

Cleaved embryos on day 2 were group-cultured (maximum of 5 embryos in each group) in 50 μL of supplemented G1 media. The groups were formed taking into consideration parameters such as evenness of blastomeres, percentage of fragmentation, cell number, and multinucleation. On the morning of day 3, embryos were washed in G-MOPS media before transfer into G2 media. The medium was refreshed daily for embryos that were undergoing extended culture for embryo transfer.

Embryo Transfer

All embryo transfers were performed under transabdominal ultrasound guidance. Patients were required to have a full bladder to facilitate ultrasonographic visualization of the cervical canal and uterine cavity.

In the study group, HETM was used after pre-equilibration at 37°C and 6% CO_2, and the embryos were cultured in HETM for 30 minutes before transfer. First, the inner sheath of the transfer catheter was rinsed with transfer medium; this was HETM (Embryoglue, Vitrolife) in the study group and was conventional medium (G2, version 3, Vitrolife) supplemented with HSA (HSA solution, Vitrolife) in the control group. Both media were pre-equilibrated at 37°C and 6% CO_2. A 50-μL Hamilton syringe (1700 series; Hamilton Bonaduz AG, Bonaduz, Switzerland) was rinsed with HETM in the study group and with G-RINSE solution in the control group. After the equipment was rinsed, the Hamilton syringe was attached to the transfer catheter, and 2 μL of air was pulled, followed by 2 μL of transfer media, 2 μL of air, 5 μL of transfer medium containing the embryos, and finally, 2 μL of air.

The clinician performing the embryo transfer procedure and the woman undergoing embryo transfer were blinded to the allocation that was effected by the nurse coordinator and conveyed to the embryologist.

Outcome Measures

Pregnancy was confirmed by measuring β-hCG levels 12 days after embryo transfer. *Clinical pregnancy* was defined as the presence of at least one gestational sac, documented with vaginal ultrasonography 2 weeks after a positive pregnancy test. *Implantation rate* was calculated as number of gestational sacs, divided by number of transferred embryos and multiplied by 100. *Ongoing pregnancy* was defined as pregnancy proceeding beyond the 16th week of gestation.

Statistical Analysis

On the basis of an expected 45% clinical pregnancy rate in the control group, in accordance with our overall results from the previous year, an a priori power calculation revealed that 537 patients would be necessary in each group to detect a 15% increase in clinical pregnancy rate with an α error of 5% and β error of 25%.

Data were analyzed by using independent samples *t*-test, Kruskal Wallis test, and analysis of variance for continuous variables and by using χ^2 and Fisher's exact tests for categorical variables. Odds ratio and 95% confidence intervals were calculated for comparison of categorical variables. The main outcome measure was the clinical pregnancy rate. Secondary outcome measures were implantation, abortion, and multiple pregnancy rates. Number-needed-to-treat (NNT) analysis

TABLE 1

Patient characteristics.

Characteristic	HETM	Control
Total no. of embryo transfer cycles	639	643
Mean female age (y)	32.8	32.9
Total no. of embryos transferred (mean)	1,718 (2.69)	1,769 (2.75)
Mean duration of infertility (y)	6.9	7.2
Mean no. of previous failed cycles	2.0	2.1
Indication for treatment, n (%)		
Male factor	273 (42.72)	261 (40.59)
Ovarian	160 (25.04)	169 (26.28)
Endometriosis	12 (1.88)	14 (2.18)
Tubal factor	78 (12.21)	85 (13.22)
Unexplained	116 (18.15)	114 (17.73)

Note: P values for comparisons of the two groups were nonsignificant across all characteristics.

Urman. Hyaluronan-enriched transfer medium. Fertil Steril 2008.

was performed for clinical pregnancy. The study population also was analyzed in subgroups according to female age, presence of previous failed attempts, and the quality of the embryos transferred.

RESULTS

Overall Outcome

A total of 1,282 embryo transfer cycles (day 3 and day 5 combined) were analyzed. Mean female age, duration of infertility, number of previous failed cycles, and indications for treatment were similar between the HETM and control groups (Table 1). Likewise, mean number of oocytes retrieved, metaphase II oocytes, two-pronuclear fertilization rate, cleavage rate, the incidence of grade 1 and 2 embryos, and the incidence of eight-cell embryos on day 3 were similar (Urman B, Yakin K, Ata B, Isiklar A, Balaban B, unpublished data). A similar number of embryos was transferred in the two groups. Implantation, clinical pregnancy, and multiple pregnancy rates were significantly increased in the HETM group (Table 2). When the data were analyzed according to subgroups, the use of HETM was associated with increased clinical pregnancy rates in women $\geq$35 years of age, in women with previous implantation failure (PIF), and in women with poor-quality embryos; however, the difference in clinical pregnancy rates did not reach statistical significance in women <35 years of age, women undergoing their first embryo transfer cycle, or women who had good-quality embryos. Implantation rates were increased in all age groups as well as for all embryos, regardless of quality.

Outcome of Day 3 Transfers

A total of 825 day 3 embryo transfer cycles was analyzed. Mean female age, duration of infertility, indications for treatment, number of oocytes retrieved, fertilization rate, cleavage rate, eight-cell embryos on day 3 and embryo grade, mean number of embryos transferred, and percentage of Grade 1 and Grade 2 embryos that were transferred were similar between the HETM and control groups (Urman B, Yakin K, Ata B, Isiklar A, Balaban B, unpublished data). Implantation, clinical pregnancy, and multiple pregnancy rates were increased with the use of HETM; however, the difference in clinical pregnancy rates did not reach statistical significance (Table 3).

Overall implantation rate of day 3 embryos, implantation rates in women $\geq$35 years of age, and implantation rates in women with PIF were significantly increased in the HETM group (Table 3).

Outcome of Day 5 Transfers

A total of 457 blastocyst transfers were analyzed. Mean female age, duration of infertility, indications for treatment,

TABLE 2

Overall outcome.

Parameter	HETM group	Control group	OR (95% CI)
CP-ET	349/639 (54.62)	312/643 (48.52)	1.28 (1.03–1.59)
Implantation rate	549/1,718 (31.96)	437/1,769 (24.70)	1.43 (1.23–1.66)
Multiple pregnancies	183 (52.44)	117 (37.50)	1.84 (1.35–2.51)
Abortion	35 (10.02)	49 (15.71)	0.60 (0.38–0.95)
CP-ET in subgroups			
Women <35 y of age	239/383 (62.40)	239/369 (64.77)	0.90 (0.67–1.22)
Women $\geq$35 y of age	110/256 (42.97)	73/274 (26.64)	2.07 (1.44–2.99)
No previous implantation failure	190/333 (57.06)	187/328 (57.01)	1.00 (0.74–1.36)
Previous implantation failures	159/306 (51.96)	125/315 (39.68)	1.64 (1.20–2.26)
Good-quality embryos[a]	285/458 (62.23)	273/471 (57.96)	1.19 (0.92–1.55)
Poor-quality embryos[b]	64/181 (35.36)	39/172 (22.67)	1.87 (1.67–2.98)
Implantation rate in subgroups			
Women <35 y of age	402/935 (42.99)	362/969 (37.36)	1.26 (1.05–1.51)
Women $\geq$35 y of age	147/783 (18.77)	75/800 (9.38)	2.23 (1.66–3.01)
No previous implantation failure	292/887 (32.92)	272/853 (31.89)	1.05 (0.86–1.28)
Previous implantation failures	257/831 (30.93)	165/916 (18.01)	2.04 (1.63–2.55)
Good-quality embryos[a]	450/1,097 (41.02)	390/1,170 (33.33)	1.39 (1.17–1.65)
Poor-quality embryos[b]	99/621 (15.94)	47/599 (7.85)	2.23 (1.54–3.22)

Note: Data are n (%) unless otherwise indicated. OR = odds ratio; CI = confidence interval; NS = not significant according to independent samples *t*-test; CP = clinical pregnancy; ET = embryo transfer.

[a] At least one G1 or G2 embryo was transferred.

[b] Only G3 and G4 embryos were transferred.

Urman. Hyaluronan-enriched transfer medium. Fertil Steril 2008.

number of oocytes retrieved, fertilization rate, cleavage rate, eight-cell embryos on day 3, cleavage-stage embryo grade, and rate of blastocyst formation were similar between the HETM and control groups (Urman B, Yakin K, Ata B, Isiklar A, Balaban B, unpublished data). Mean number of blastocysts that were transferred and blastocyst quality were likewise similar (Table 4). Implantation and multiple pregnancy rates were significantly increased with the use of HETM. The differences in clinical pregnancy and abortion rates were, however, not statistically significant (Table 4).

Blastocyst transfer in HETM significantly increased the clinical pregnancy rate in women >35 years of age and in women with PIFs. Although the clinical pregnancy rate after transfer of poor-quality blastocysts was increased by approximately 13%, the difference did not reach statistical significance. The implantation rate was significantly increased with the use of HETM in women who were ≥35 years of age and in women with PIFs. Hyaluronan-enriched transfer medium increased the implantation efficiency of both good- and poor-quality blastocysts.

Number-Needed-to-Treat Analysis

When HETM was used for every woman undergoing embryo transfer, 17 patients had to undergo embryo transfer with HETM for achievement of one additional pregnancy. The beneficial effect of HETM was more evident for women who were ≥35 years of age, women with PIFs, and women who had only poor-quality embryos; NNT for one additional pregnancy ranged between 7 and 10 in these subgroups. When blastocyst transfers were undertaken with HETM in women who were ≥35 years of age, one additional clinical pregnancy was achieved in every four transfers. Number-needed-to-treat analyses in subgroups in which clinical pregnancy rates were significantly different are given in Table 5.

DISCUSSION

Implantation is a delicate process involving complex interactions of various factors that were derived either from the embryo or endometrium (17–20). Over the years, noteworthy progress has been achieved in the success rates of assisted reproductive techniques; however, embryo implantation still remains a major limiting factor.

In the first published study with an HETM in human beings, Schoolcraft et al. (21) reported that significantly higher implantation rates were associated with the use of a HETM. In a later preliminary study that used HETM, we showed increased implantation rates in a subgroup of patients who had previously experienced repeated implantation failures (22). Our present study further demonstrated that the use of an

TABLE 3

Outcome of day 3 embryo transfer cycles according to specific patient and cycle characteristics.

Parameter	HETM	Control	OR (95% CI)
No. of embryo transfer cycles	412	413	
No. of embryos transferred (mean)	1,252 (3.04)	1,296 (3.14)	NS
CP-ET	188/412 (45.63)	165/413 (39.95)	1.26 (0.96–1.66)
Implantation rate	309/1,252 (24.68)	242/1,296 (18.67)	1.43 (1.18–1.73)
Multiple pregnancies	104 (55.32)	69 (41.82)	1.72 (1.13–2.63)
Abortion	19 (10.11)	26 (15.76)	0.60 (0.32–1.13)
CP-ET in subgroups			
Women <35 y of age	118/226 (52.21)	116/218 (53.21)	0.96 (0.66–1.39)
Women ≥35 y of age	70/186 (37.63)	49/195 (25.13)	1.80 (1.16–2.79)
No previous implantation failure	105/227 (46.26)	101/224 (45.09)	1.05 (0.72–1.52)
Previous implantation failures	83/185 (44.86)	64/189 (33.86)	1.59 (1.05–2.41)
Good-quality embryos[a]	152/303 (50.17)	144/310 (46.45)	1.16 (0.85–1.59)
Poor-quality embryos[b]	36/109 (33.03)	21/103 (20.39)	1.93 (1.03–3.59)
Implantation rate in subgroups			
Women <35 y of age	221/619 (35.70)	196/633 (30.96)	1.24 (0.98–1.57)
Women ≥35 y of age	88/633 (13.9)	46/663 (6.94)	2.17 (1.49–3.15)
No previous implantation failure	163/670 (24.33)	160/660 (24.24)	1.00 (0.78–1.29)
Previous implantation failures	146/582 (25.09)	82/636 (12.89)	2.26 (1.68–3.05)
Good-quality embryos[a]	256/785 (32.61)	222/847 (26.21)	1.36 (1.10–1.69)
Poor-quality embryos[b]	53/467 (11.35)	20/449 (4.45)	2.75 (1.61–4.67)

Note: Data are n (%) unless otherwise indicated. OR = odds ratio; CI = confidence interval; NS = not significant according to independent samples *t*-test; CP = clinical pregnancy; ET = embryo transfer.

[a] At least one G1 or G2 embryo was transferred.

[b] Only G3 and G4 embryos were transferred.

Urman. Hyaluronan-enriched transfer medium. Fertil Steril 2008.

TABLE 4

Outcome of day 5 embryo transfer cycles according to specific patient and cycle characteristics.

Parameter	HETM	Control	OR (95% CI)
No. of blastocyst transfer cycles	227	230	
No. of embryos transferred (mean)	466 (2.05)	473 (2.06)	NS
CP-ET	161/227 (70.93)	147/230 (63.91)	1.38 (0.93–2.04)
Implantation rate	240/466 (51.50)	195/473 (41.23)	1.51 (1.17–1.96)
Multiple pregnancies	79 (49.07)	48 (32.65)	1.99 (1.25–3.16)
Abortion	16 (9.94)	23 (15.65)	0.59 (0.30–1.18)
CP-ET in subgroups			
Women <35 y of age	121/157 (77.07)	123/151 (81.46)	0.77 (0.44–1.33)
Women ≥35 y of age	40/70 (57.14)	24/79 (30.38)	3.06 (1.56–5.99)
No previous implantation failure	85/106 (80.19)	86/104 (82.69)	0.85 (0.42–1.70)
Previous implantation failures	76/121 (62.81)	61/126 (48.41)	1.80 (1.08–2.99)
Good-quality embryos[a]	133/155 (85.81)	129/161 (80.12)	1.50 (0.83–2.71)
Poor-quality embryos[b]	28/72 (39.22)	18/69 (26.32)	1.80 (0.88–3.69)
Implantation rate in subgroups			
Women <35 y of age	181/316 (57.28)	166/336 (49.40)	1.37 (1.01–1.87)
Women ≥35 y of age	59/150 (39.33)	29/137 (21.17)	2.41 (1.43–4.08)
No previous implantation failure	129/217 (59.45)	112/193 (58.03)	1.06 (0.72–1.57)
Previous implantation failures	111/249 (44.58)	83/280 (29.64)	1.91 (1.33–2.73)
Good-quality embryos[a]	194/312 (62.18)	168/323 (52.01)	1.52 (1.11–2.08)
Poor-quality embryos[b]	46/154 (29.87)	27/150 (18.00)	1.94 (1.13–3.33)

Note: Data are n (%) unless otherwise indicated. OR = odds ratio; CI = confidence interval; NS = not significant according to independent samples *t*-test; CP = clinical pregnancy; ET = embryo transfer.

[a] At least one good-quality blastocyst was transferred.

[b] Only poor-quality blastocysts were transferred.

Urman. Hyaluronan-enriched transfer medium. Fertil Steril 2008.

HETM increased implantation and clinical pregnancy rates, not only in women with PIFs but in general. The beneficial effect of HETM with regard to implantation rate was evident in women who received either cleavage-stage embryos or blastocysts. When cleavage-stage embryo and blastocyst transfer cycles were analyzed in combination, abortion rates were also decreased in women who received HETM. The lack of statistical significance for differences in clinical pregnancy rates when day 3 and day 5 cycles were analyzed separately is most probably a result of the effect of diminished sample sizes in subgroup analysis. Clinical pregnancy rates were consistently higher by >5% in the total study population and in the subgroups of day 3 and day 5 transfers, in favor of HETM. A consistent 5% increase can be regarded as clinically important despite lack of statistical significance, which could be solely dependent on the sample size of the study and event rates in the groups. As evident from the 95% confidence intervals, these differences could have reached statistical significance, had the sample size been larger. However, the study was powered to detect a 15% absolute difference between clinical pregnancy rates.

Recently, Valojerdi et al. (14) reported improved clinical outcome after use of HETM. The improvements were noted mainly in patients with tubal-factor infertility and in couples who had had recurrent implantation failures. That study, however, was not properly randomized, and hence, potential sources of bias may have been introduced. In another study, Loutradi et al. (15) reported no difference in total pregnancy rate in the group of patients who had their embryos transferred in a medium containing a high concentration of hyaluronan, compared with patients undergoing embryo transfer using conventional embryo transfer medium. That study, however, was retrospective and nonrandomized; implantation rates were not given, and overall pregnancy rates were very low, despite the transfer of three or four embryos in a majority of patients and the use of a mixture of embryo culture mediums (15).

Although the major biological functions of hyaluronan are still unknown, various mechanisms can be proposed for its beneficial effect on implantation. Hyaluronan may have direct and indirect effects on the embryo and its implantation potential.

Hyaluronic acid is one of the most abundant glycosaminoglycans in the uterine, oviductal, and follicular fluids in mouse, pig, human beings, and cattle. It is a major component of the extracellular matrix of tissues that exhibit rapid growth and regeneration. Hyaluronic acid action is partly mediated

by its relationship with CD44, its major cell surface receptor (23). CD44 is expressed in human embryos from one- to eight-cell stages (9). Staining of archival paraffin–embedded cell blocks for hyaluronic acid and its receptor CD44 showed a peak during the secretory phase that is coincident with the period in which the endometrium is most receptive to embryo implantation (23).

Hyaluronan may directly stimulate the growth of the embryo or provide a more appropriate environment for the embryo to be nourished by the supplements. Surface receptors for hyaluronan were detected on human and bovine embryos from the oocyte to the blastocyst stage (24). Hyaluronan may confer some protection to the embryos during in vitro growth and cryopreservation. Lane et al. (25) showed that addition of hyaluronan to the culture medium containing either bovine serum or recombinant albumin increased the ability of blastocysts to survive cryopreservation. Similarly, Stojkovic et al. (26) compared the developmental capacity of bovine embryos in synthetic oviduct fluid culture medium containing either bovine serum albumin or hyaluronan. The addition of hyaluronan significantly increased the rate of blastocyst development. The percentage of eight-cell embryos that re-expanded and hatched after freezing and thawing also was higher for embryos that were cultured in the presence of hyaluronan. Supplementing culture media with 0.5 mg/mL of hyaluronic acid and 1.0 mg/mL of heparin significantly increased blastocyst formation rate compared with the control group, suggesting an embryotropic effect of hyaluronic acid on the in vitro growth of porcine embryos (27).

Cell–cell adhesion and cell–matrix adhesion have been shown to be increased by hyaluronan (28). This may facilitate the apposition and attachment of the blastocyst (11). Furthermore, hyaluronan can promote angiogenesis by both its degradation products and by interaction with epidermal growth factor. Several studies have demonstrated increased angiogenesis after administration of hyaluronan (29, 30). Hyaluronan and its receptor CD44 have been implicated both in angiogenesis and tumorigenesis in the human endometrium (31). Trophoblast invasion may be facilitated by hyaluronan in the same manner.

Hyaluronan potentially could facilitate cell migration and proliferation. Deposition of high concentrations of hyaluronan results in an expansion, or "loosening", of spaces within the connective tissue (32). Conversely, the removal of hyaluronan was associated with a dramatic shrinkage of the tissue and was coincident with loss of tissue hydration (33). Thus, the hydrodynamic effects of hyaluronan, such as the hydration and subsequent expansion that occurs in the presence of a high–molecular mass, negatively charged polysaccharide, often were suggested as the primary mechanism for how hyaluronan may facilitate cell migration and/or proliferation. Hyaluronan involvement in cell migration was somehow cell specific, serving as a favorable milieu for some cell types and as an impediment for others, possibly as a result of the presence or absence of cell-surface hyaluronan receptors (34–36).

TABLE 5

Number needed to treat to achieve one additional clinical pregnancy with use of HETM in groups that had statistically significant improvements in clinical pregnancy rates.

Parameter	NNT
Overall	
For 1 additional clinical pregnancy in any women	17
For 1 additional clinical pregnancy in women >35 y of age	7
For 1 additional clinical pregnancy in women with PIF	7
For 1 additional clinical pregnancy in women who have no good-quality embryos	8
Day 3 transfers	
For 1 additional clinical pregnancy in women >35 y of age	8
For 1 additional clinical pregnancy in women with PIF	10
For 1 additional clinical pregnancy in women who have no good-quality embryos	8
Day 5 transfers	
For 1 additional clinical pregnancy in women >35 y of age	4
For 1 additional clinical pregnancy in women with PIF	7

Urman. Hyaluronan-enriched transfer medium. Fertil Steril 2008.

Hyaluronan may have a role in the preparation of the endometrium for embryo implantation because it increases significantly on the day of implantation in the mouse uterus and appears to be associated with regions that contain stromal cells that are proliferating in preparation for embryo implantation (37). Human embryos express the surface receptor for hyaluronan, CD44, throughout development from the oocyte to the blastocyst stage (24). The changing levels of hyaluronan in the endometrium may correlate with the reorganization of cellular proliferation, differentiation, and tissue breakdown, along with alterations in the composition of the extracellular matrix.

Alternatively, the beneficial effect of hyaluronan in the transfer medium may be a physical phenomenon, which acts by facilitating rapid diffusion of the contents of the transfer medium (the embryo). Hyaluronan is a viscous sticky solution that more closely resembles the natural secretions

of the uterus. Because uterine fluid is a viscous solution, the transfer of a relatively aqueous solution, such as culture medium with albumin or serum, to the uterine lumen will result in the slow dispersal of the medium and embryo with the luminal contents.

Our findings suggest that the effects of HETM on in utero embryo growth or on embryo–endometrium cross-talk can be more important than its effects on the endometrium. Because the implantation and multiple pregnancy rates increased more prominently than did clinical pregnancy rates with the use of HETM, we suppose that in the presence of a favorable endometrium, HETM increases the implantation potential of transferred embryos. The observed increased implantation rates in older women and in women with poor-quality embryos also support our hypothesis. A woman's age is known to affect oocyte and embryo quality, rather than the endometrium. The use of HETM in this group of women most likely increases implantation by its effects on the embryo.

We can conclude that the enrichment of the transfer medium with hyaluronan may benefit couples undergoing assisted reproduction with the transfer of cleavage-stage embryos or blastocysts. Overall implantation and clinical pregnancy rates were significantly increased with the use of HETM. Decreasing the number of transferred embryos should be considered to avoid multiple pregnancies whenever HETM is used. A beneficial effect was evident particularly in women >35 years of age, in women with PIF, and in women receiving poor-quality embryos.

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