Integrating insulin into single-step culture medium regulates human embryo development in vitro

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Objective: To evaluate the effect of supplementing single-step embryo culture medium with insulin on human embryo development.
Design: Comparative study.
Setting: Two private centers.
Patient(s): The study involved a sibling oocyte split of 5,142 retrieved oocytes from 360 patients.
Intervention(s): Sibling oocytes split after intracytoplasmic sperm injection for culture from day 0 through day 5 or 6 in insulin-supplemented or control medium. Women were split to receive their embryos from insulin-supplemented or control medium.
Main Outcome Measure(s): Clinical pregnancy rate.
Result(s): There were significantly higher rates of clinical, ongoing, and twin pregnancies in the insulin-supplemented arm than in the control arm. On day 3, embryo quality and compaction were higher in insulin-supplemented medium. On day 5, insulin supplementation showed higher rates of blastocyst formation, quality, and cryopreservation.
Conclusion(s): Insulin supplementation of single-step embryo culture medium from day 0 through day 5 or 6 improved clinical pregnancy rate and human embryo development. However, these findings need further confirmation through a multicenter randomized controlled trial that may include other patient populations and different culture media. (Fertil Steril® 2017;107:405–12. ©2016 by American Society for Reproductive Medicine.)
Key Words: Embryo culture media, insulin embryonic effect, blastocyst

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Despite significant strides over the past four decades, success of in vitro fertilization (IVF) remains relatively elusive, and the benchmark varies among clinics and around the world. An optimistic analysis reported cumulative live birth rates of up to 86% after six cycles of IVF in young good-prognosis women (1). In another study, women younger than 40 years achieved a cumulative live birth rate of 68% after six cycles, but only 32% for the first cycle (2). However, patient retention through six cycles to achieve this high live birth rate is challenging. Suboptimal embryo culture condition contributes to this relative inefficiency with myriad factors. These include, but are not limited to, pH, temperature, incubator O_2 level, volatile organic compounds, incubator type, and in particular, culture medium (3–7).

Culture medium is a complex solution that comprises a range of elements to provide the embryo with hydration, ions, and nutrients while maintaining a homeostatic and relatively nonstressful environment. Over the past decades, endeavors to determine the optimal medium have followed two main approaches: the "sequential media" paradigm which provides the developing embryo with stage-specific nutrients, and "single-step" medium formulation that provides all of the required nutrients continuously (8). However, to date, neither approach has produced a clearly superior medium (9, 10). Using unconditioned culture media, which contain embryotrophic factors, such as cytokines, steroid hormones, growth factors, and insulin, results in better preimplantation embryo development in vitro (11–14). Thus, the supplementation of culture media with specific embryotropic factors may provide better support for embryo development in vitro.

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Of particular interest is the hormone insulin. Insulin tends to increase cell proliferation and differentiation and decreases apoptosis of in vitro cultured embryos (15). In a mouse model, insulin supplementation of culture medium yielded a 25% increase in the inner cell mass (ICM) and increased compaction and blastulation rate, and conversely the lack of insulin resulted in decreased embryo development and cell proliferation (16–19). This suggests that embryonic growth may be sensitive to insulin levels. Although the exact mechanism by which insulin influences embryo development remains unclear, oocytes and preimplantation embryos express insulin receptor genes in several species, including humans (20, 21). In human embryonic stem cell culture, insulin supplementation yields to increase epiblast cell numbers but not ICM, suggesting a particular role in differentiating ICM toward more pluripotent cells rather than a general mutagenic activity (22). Furthermore, a recent study that used a high concentration of insulin in the culture medium successfully cultured human embryos in vitro to day 13 independently from any maternal tissue contact (23).

With this background in mind, the aim of the present comparative study was to determine whether insulin supplementation of a single-step culture medium would have an influence on and embryo development in vitro and pregnancy rate.

MATERIALS AND METHODS

From January 2015 to December 2015, two private IVF centers conducted this prospective comparative study. The research ethics committees of the two centers "IbnSina Center (Sohag, Egypt) and Banoon Center (Assiut, Egypt)" approved the protocol (Ethical Clearance no. 005-2014).

Stage 1 ("allocation I") involved a sibling oocyte split between insulin-supplemented and control media; stage 2 ("allocation II") of the trial involved a patient split between transferred embryos from insulin-supplemented and control media.

Women included in this study were 18–37 years of age with body mass index (BMI) ≤ 31 kg/m^2 and normal response to stimulation with ten or more follicles (seven or more mature oocytes expected) as well as normal endometrial thickness (8–12) and echographic pattern at the time of the hCG trigger. They were undergoing their first treatment cycle or had had a previous successful treatment cycle. The study only included fresh semen samples with a total concentration of 10×10^6/mL and 5% progressive motility, excluded globozoospermia and pinhead samples, and did not use any other morphologic criteria.

In stage 1, an embryologist that not involved in the study design split each woman's inseminated oocytes (all subjects underwent intracytoplasmic sperm injection [ICSI]) evenly between each of two dishes that were unlabeled regarding the containing medium, which was either insulin-supplemented or control medium. For odd numbers of injected oocytes, the last oocyte was assigned randomly to one of the treatments. Color codes were used to label the culture dishes to differentiate between insulin-supplemented and control medium. Oocyte quality was not a factor in assigning oocytes. According to the Istanbul consensus, embryologists graded and recorded the oocyte and embryo quality (24).

For stage 2, 360 women were enrolled to receive embryos on day 5 from either the treatment or the control group according to a list generated with the use of a computer program (Excel). On the day of oocyte retrieval, a research counselor sent a sealed opaque envelope that contained the results of assignment to the laboratory. On the day of embryo transfer, a research counselor opened the sealed opaque envelope and passed the result to the laboratory director. The two highest-quality blastocysts from the insulin-supplemented medium and the control medium were each placed in labeled droplets (coded). The decision regarding transfer of embryos from either the insulin-supplemented arm or the control arm was received from the laboratory director, based on the result of the sealed envelope.

Ovarian Stimulation Protocol and Luteal Phase Support

A midluteal pituitary down-regulation protocol was used for all women. GnRH agonist (Decapeptyl, 0.1 mg; Ferring) was started on day 21 of the cycle preceding treatment and continued throughout the next cycle. On cycle day 2, quiescent ovaries were confirmed by laboratory and ultrasound examinations. Gonadotropin injection (recombinant FSH [Puregon; MSD] and hMG [Menogon; Ferring]) for multifollicular ovarian stimulation was begun at a dose of 150–300 IU per day and was continued throughout the cycle. An ultrasound scan was done every other day, starting on day 5 of stimulation. When 3 follicles ≥ 18 mm were seen on ultrasound, a 10,000 IU hCG trigger injection (Choriomon; IBSA) was given for final oocyte maturation. For luteal phase support, intramuscular P (100 mg/mL, Prontogest; IBSA) was started on the day after retrieval ("day 1") and continued up to 8 weeks of gestation.

Sperm Preparation, Oocyte Retrieval, Denudation, and ICSI

Semen samples were prepared through a density gradient, according to the World Health Organization manual (25) (except for diluting gradient with buffer) with the use of Puresperm (Nidacon). The pellet was washed twice and then maintained at room temperature in HEPES-buffered medium (Allgrade Wash, Life Global). Ultrasound-guided oocyte retrieval was performed 36 hours after hCG administration, and each follicle was aspirated into 1 mL HEPES-buffered medium (Global/HEPES; Life Global) with aspirates handled at 37°C with the use of tube warmers. Oocyte denuding was performed 1 hour after collection with the use of hyaluronidase (Life Global) and mechanical aid (denudation pipettes; Vitrolife). Only metaphase II (MII) oocytes were injected 4 hours after denudation in Global/HEPES medium according to Palermo et al. (26).

Culture Protocol and Embryo Scoring

The insulin-supplemented medium was freshly prepared the day before oocyte retrieval and ICSI. Insulin (10 mg/mL, I9278-5ML; Sigma) was diluted at 1:200,000 (v/v) with culture medium (Global [5 mg/mL human serum albumin (HSA)]; Life Global), to give 50 ng insulin per mL of medium. The 1:200,000 (v/v) dilution of insulin to primary medium was unlikely to cause a deleterious effect. Insulin batches were tested with the use of the 1-cell mouse embryo assay to exclude the possibility of embryotoxicity; only batches of insulin that resulted in $\geq$90% expanded blastocysts were used. Culture dishes of 20 μL medium per droplet were prepared, and the droplets were overlaid by 5 mL oil (Ovoil; Vitrolife). The prepared dishes were then incubated overnight at pH 7.25 $\pm$ 0.02 (7.5% CO_2; 5% O_2; 87.5% N_2) in C-Top incubators (Labotect).

Each woman's injected oocytes were divided between control ("Global/HSA") medium and insulin-supplemented Global/HSA medium and maintained in culture for 5–6 days. Each medium droplet contained three to four oocytes. Fertilization checks and embryo grading were done according to the Istanbul consensus (24). High-quality cleavage-stage embryos were defined as having 4 cells on day 2 or 7–8 cells on day 3, with symmetric blastomeres and $<$10% fragmentation by volume. High-quality blastocysts were defined as those with rounded and dense ICM with many twin trophectoderm cells creating a connected zone and a blastocele cavity of $>$100% by volume on day 5 ($\geq$3.1.1 grade, Istanbul consensus) (24).

Embryo Transfer

Up to two blastocysts were transferred on day 5 to each woman (excluding women with reduced uterine cavity or previous preterm birth, for whom only one blastocyst was transferred). The decision to transfer embryos from the insulin-supplemented or control group was based on the result of the closed envelope. Under ultrasound guidance, the Sydney IVF Transfer Set (Cook) was used to transfer embryos in up to 30 μL Embryoglue (Vitrolife). The remaining blastocysts were vitrified either on day 5 if expansion was grade $\geq$3.1.1 or after culture to day 6. All women were advised to have a serum β-hCG titer performed 14 days after embryo transfer, and when a test was positive an ultrasound confirmation was carried out 4–5 weeks after embryo transfer.

Study Outcomes

The study's primary outcome was clinical pregnancy rate, defined as the presence of a positive fetal heart beat on ultrasound $\geq$4 weeks after embryo transfer. Prespecified secondary outcomes were the rates of blastocyst formation (formed blastocysts per fertilized oocyte) and high-quality blastocysts (formed blastocysts of grade $\geq$3.1.1 per fertilized oocyte). Other recorded outcomes were the rates of ongoing pregnancy (defined as number of pregnancies after 20 weeks of gestation) and implantation (number of positive heart beats on ultrasound per embryo transferred). In addition, the rates of high-quality cleavage-stage embryos, compaction (embryos with disappearing blastomere boundaries per fertilized oocytes) on day 3, and fertilization (fertilized oocytes with two pronuclei per MII oocyte injected) were also recorded.

Statistical Analysis

An increase of 15% clinical pregnancy rate (from 38% to 53%) was calculated as the minimally important difference of using insulin-supplemented medium compared with control medium. The 38% was based on analyzing the conservative average of the success rate in the preceding 1,000 cycles in the centers in which the study was conducted, and the 15% difference was chosen after consulting assisted reproductive technology scientists. To detect this difference, 340 women would have to be randomly assigned into two groups (170 for transfer of embryos from the insulin-supplemented medium, and 170 for transfer of embryos from the noninsulin-supplemented medium) to provide 80% power at an α of 5%. Anticipating 5% dropout, the numbers of participants needed would be 357 in total (27). To calculate the significance, the risk ratio (RR) with 95% confidence interval (CI) was calculated by means of binomial logistic regression with the use of log link for the dichotomous outcomes. The continuous variables were tested for normality by means of Shapiro-Wilk test. The nonnormally distributed variables were analyzed with the use of Mann-Whitney *U* test and Wilcoxon signed rank test. For the normally distributed variables, Student *t* test was used whenever indicated. Chi-square and Fisher exact tests were used to compare the proportions for infertility causes. Data are presented as median (interquartile range [IQR]), mean $\pm$ SD, and proportions. *P* values of $<$.05 were deemed to indicate statistical significance. We used SPSS version 21.0 for all statistical analyses.

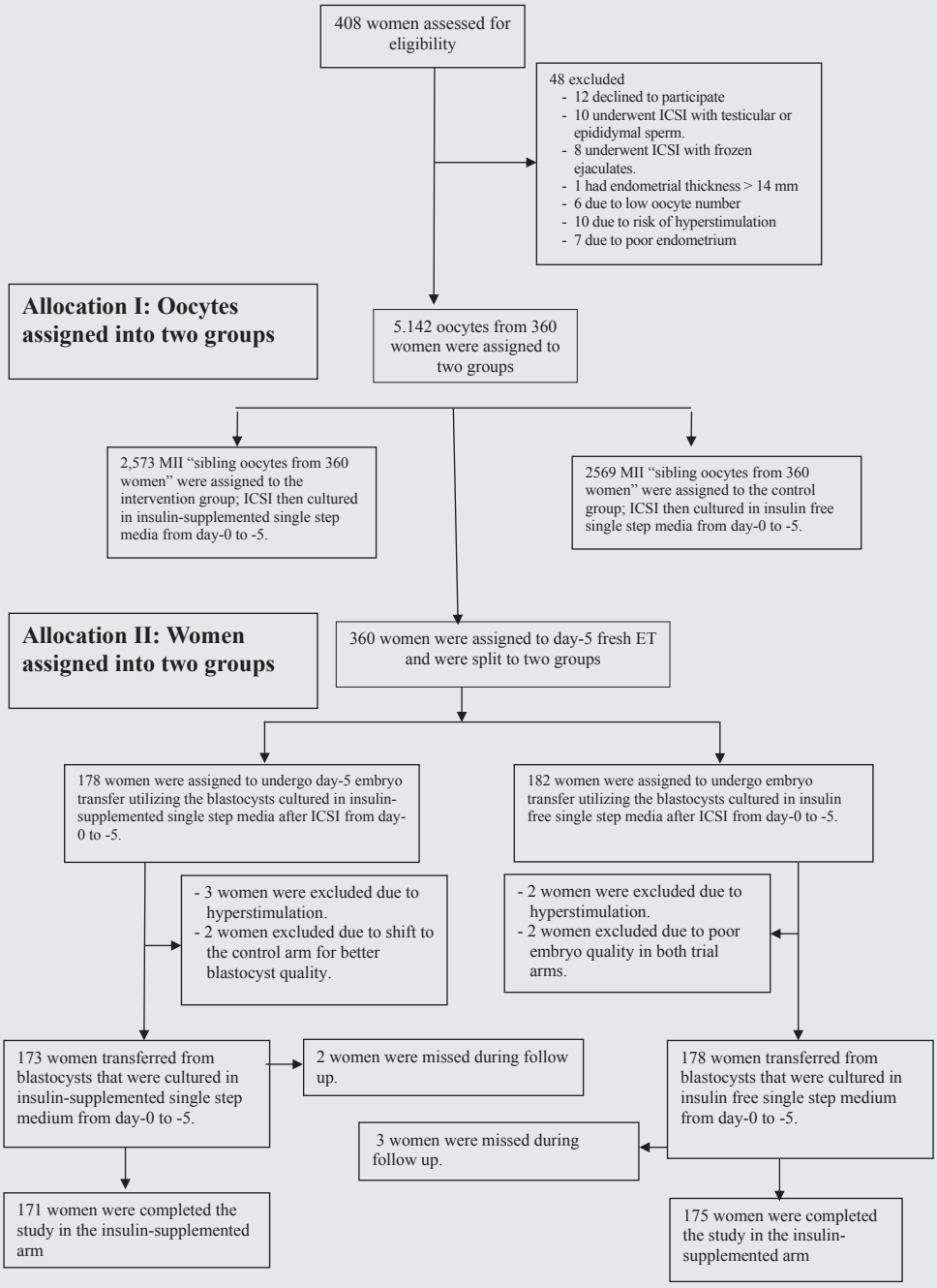
RESULTS

From January 2015 to December 2015, 408 women were approached, of whom 360 were eligible to participate in the study (Fig. 1). Fourteen patients were excluded from the primary outcome analysis but were still included in the embryo development analysis. Of these, five did not receive a transfer because of hyperstimulation syndrome, two did not receive a transfer because of poor embryo quality, two were shifted to transfer embryos from the control medium rather than the insulin-supplemented medium owing to higher blastocyst quality, and the remaining five were lost follow-up.

ICSI was performed on 5,142 MII oocytes from 360 women, and all of the injected oocytes were washed in Global/HSA (Life Global). For the stage 1 allocation, the injected MII oocytes were assigned immediately after ICSI with the use of the "sibling oocyte split" approach: 2,573 injected MII oocytes were cultured in insulin-supplemented medium and 2,569 injected MII oocytes in control medium (Fig. 1).

For the stage 2 allocation, the 360 women were split into two groups, 178 in the intervention group and 182 in the control group to receive transfer embryos from either the insulin-supplemented or the control medium. Of these, 171 women in the insulin-supplemented group and 175 in the control group completed the study (Fig. 1).

FIGURE 1



Allocation I refers to stage 1 of the study, in which the mature oocytes were split to insulin-supplemented or control medium. Allocation II refers to stage 1 of the study, in which women were split to undergo transfer of embryos that were cultured in insulin-supplemented or control medium. ICSI = intracytoplasmic sperm injection; ET = embryo ransfer.

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The patients' demographic and baseline characteristics identified no significant differences between groups in median age, BMI, mean the number of oocytes collected, previous ICSI attempts, number of embryos transferred, basal FSH level, antral follicle count, or total FSH/hMG dose (Table 1). The median number of months of trying to conceive was greater in the intervention group (22.0 mo, IQR 14.0–36.0) than in the control group (19.0 mo, IQR 13.0–32.0; P=.022), but there was no significant difference in the causes of infertility.

Although both groups had similar rates of fertilization and embryo cleavage, other embryo development parameters were significantly improved in the intervention group (Table 2). The rates of high-quality embryos and embryo compaction on day 3 were significantly higher in the

TABLE 1

Baseline characteristics by trial group.

Characteristic	Intervention (n = 180)	Control (n = 180)	*P* value
Age (y)	27.5 (25–31)	27 (25–31)	NS
BMI (kg/m^2)	28 (26–30)	28 (27–29)	NS
Time attempting to conceive (mo)	22 (14–36)	19 (13–32)	.022
No. of previous ICSI attempts	1.09 ± 0.34	1.1 ± 0.37	NS
Basal FSH (IU/L)	6.4 ± 2.5	6.5 ± 2.7	NS
Antral follicle count	13 ± 6	14 ± 7	NS
Total FSH/hMG	2,835 ± 1,545	2,771 ± 1,488	NS
Infertility cause			NS
PCOS	30 (17%)	23 (13%)	
Male	39 (22%)	45 (25%)	
Tubal	58 (32%)	56 (31%)	
Uterine	53 (29%)	56 (31%)	
No. of oocytes collected	15.2 ± 3.7	15.6 ± 3.6	NS
No. of embryos transferred	1.94 ± 0.24	1.92 ± 0.27	NS

Note: Differences between the groups were compared by means of Mann-Whitney *U* test for median (interquartile range), Student *t* test for mean ± SD, and chi-square or Fisher exact test for n (%), as appropriate. There were no significant differences between the groups except for the median time attempting to conceive (P=.022). BMI = body mass index; ICSI = intracytoplasmic sperm injection; NS = nonsignificant; PCOS = polycystic ovary syndrome.

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intervention group (RR 1.15 [95% CI 1.11–1.19; P<.0001] and RR 2.42 [95% CI 2.19–2.69; P<.0001], respectively). Similarly, there were higher rates of embryos with blastocyst formation and high-quality blastocysts in the intervention group (RR 1.35 [95% CI 1.27–1.43; P<.0001] and RR 1.82 [95% CI 1.68–1.99; P<.0001], respectively). As might be expected from these outcomes, there was also a higher rate of blastocyst cryopreservation in the intervention group (RR 1.53, 95% CI 1.41–1.66; P<.0001).

A total of 346 women reached the study primary end point analysis: 171 in the intervention group and 175 in the control group (Fig. 1). Although both groups had similar overall pregnancy, chemical pregnancy, miscarriage, and implantation rates (RR 1.14 [95% CI 0.94–1.37; P=.19], RR 0.65 [95% CI 0.34–1.23; P=.18], RR 0.50 [95% CI 0.23–1.11; P=.08], and RR 1.19 [95% 0.87–1.63; P=.26], respectively; Table 3), there was a significantly higher clinical pregnancy rate in the intervention group (87/171, 51%) than in the control group (69/175, 39%; RR 1.29, 95% CI 1.02–1.63; P=.03). Similarly, the ongoing pregnancy rate was significantly higher in the insulin-supplementation group (78/171, 46%) than in the control group (55/175, 31%; RR 1.44, 95% CI 1.10–1.90; P=.008). Of note, the twin pregnancy rate was significantly higher in the intervention group (19/171, 11%) than in the control group (5/175, 3%; RR 3.9, 95% CI 1.48–10.18; P=.003).

The multivariate logistic regression analysis identified no correlation between cycle characteristics, patient demographics, and clinical variables and the clinical pregnancy rate, except for BMI and insulin supplementation. In the intervention group, BMI significantly decreased clinical pregnancy rate (adjusted odds ratio [OR] 0.89, 95% CI 0.79–0.99; P=.032), and insulin supplementation significantly improved clinical pregnancy rate (adjusted OR 1.60, 95% CI 1.03–2.49; P=.037).

DISCUSSION

Insulin has been shown to improve mouse embryonic development in vitro when included in embryo culture media (16, 18, 19). The present comparative study suggests that insulin regulates human embryo development as well and may provide an avenue for improving human embryo

TABLE 2

Embryologic outcomes by trial group.

Outcome	Intervention (n = 360)	Control (n = 360)	Risk ratio (95% CI)	*P* value
Fertilization rate/injected MII oocytes	2,041/2,573 (79%)	2,016/2,569 (78%)	1.01 (0.98–1.04)	.3
Cleavage rate/fertilized oocytes	2,036/2,041 (99%)	2,008/2,016 (99%)	1.00 (0.99–1.01)	.39
Top-quality day 2 and 3 embryos/fertilized oocytes	1,664/2,041 (82%)	1,428/2,016 (71%)	1.15 (1.11–1.19)	<.0001
Compaction rate/fertilized oocytes	944/2,041 (46%)	384/2,016 (19%)	2.42 (2.19–2.69)	<.0001
Blastocyst formation rate/fertilized oocytes	1,274/2,041 (62%)	934/2,016 (46%)	1.35 (1.27–1.43)	<.0001
Top-quality blastocysts/fertilized oocytes	1,014/2,041 (50%)	536/2,016 (27%)	1.82 (1.68–1.99)	<.0001
Cryopreservation rate/fertilized oocytes	925/2,041 (45%)	598/2,016 (30%)	1.53 (1.41–1.66)	<.0001

Note: Differences between the groups were compared by means of logistic regression analysis. CI = confidence interval; MII = metaphase II.

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TABLE 3

Clinical outcomes by trial group.

Outcome	Intervention	Control	Risk ratio (95% CI)	*P* value
Overall pregnancy rate (%)	101/171 (60%)	91/175 (52%)	1.14 (0.94–1.37)	.19
Clinical pregnancy rate (%)	87/171 (51%)	69/175 (39%)	1.29 (1.02–1.63)	.03[a]
Chemical pregnancy rate (%)	14/171 (8%)	22/175 (13%)	0.65 (0.34–1.23)	.18
Ongoing pregnancy rate (%)	78/171 (46%)	55/175 (31%)	1.44 (1.10–1.90)	.008[a]
Twin pregnancy rate (%)	19/171 (11%)	5/175 (3%)	3.9 (1.48–10.18)	.003[a]
Implantation rate (%)	128/352 (36%)	112/346 (32%)	1.19 (0.87–1.63)	.26

Note: Differences between the groups were compared by means of logistic regression analysis. CI = confidence interval.
[a] $P < .05$.

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culture in vitro beyond the current standards, based on our observation that insulin-supplemented culture medium improved embryo development and quality and increased the clinical pregnancy rate.

Several studies have analyzed existing data to identify a notably superior embryo culture medium. Most recently, a Cochrane review by Youssef et al. showed insufficient evidence to identify a superior medium (10), likely because numerous factors besides culture medium also affect embryo development in the laboratory. In addition, improper study design can often preclude drawing concrete conclusions.

It is known that current embryo culture media lack significant factors that are present in human tubal and uterine fluids, such as growth factors, hormones, and cytokines. Growth factors, including insulin, have pleiotropic and mutagenic effects on cells, including the preimplantation embryo (16, 28, 29). Insulin has been shown to have a positive impact on embryo compaction, blastocyst formation, ICM, and ICM differentiation to epiblast (15–19,22). Interestingly, culturing rat embryos in insulin-depleted serum resulted in a decrease in embryonic growth, which was restored by the addition of insulin, with no evidence of harm from insulin supplementation at concentrations of up to 10,000 μg/mL (30, 31). This is likely one reason for impaired cleavage-stage embryo development in the presence of high glucose concentrations (32).

Our study was designed to determine whether the addition of insulin to human embryo culture medium would have effects similar to those reported in nonhuman studies. We took into consideration the findings of Meintjes et al. that showed better outcomes after adding Synthetic Serum Substitute (SSS) rather than HSA to embryo culture medium, presumably because of the presence of a range of embryotrohic factors in SSS, including insulin (11, 12). We were also encouraged about the importance of our study by the new medium formulation by Patrick Quinn (Advantage Protein Plus Blastocyst Medium with Insulin; Sage), which contains insulin.

Because culture media manufacturers do not routinely provide information about the composition of their products, it can be difficult to determine whether a medium contains insulin. Based on the analyses conducted by Morbeck et al., we were able to confirm that the medium we used in the present study (Global) was indeed free of insulin (14). In addition, we supplemented the culture medium with HSA rather than SSS or similar because the level of embryotropic contamination in HSA is undetectable. However, we do not have positive evidence that the HSA product used to supplement the medium was completely devoid of insulin.

Insulin has been detected in Cook media at 347 pg/mL, and in Blastassist (Origio) at up to 273 μg/mL (14), and recently up to 10 mg/mL was used to culture post-blastocyst human embryos (33). We measured the insulin level of Quinn's Advantage Protein Plus Blastocyst Medium with Insulin in our laboratories (unpublished data) and found it to be close to 60 ng/mL. We chose to use an insulin concentration of 50 ng/mL for our study because it was close to Quinn's medium level, and between the very low level of Cook media and the very high level of Blastassist.

In our study, the baseline characteristics were balanced between the two groups, apart from the longer time attempting to conceive for the intervention group. This is unlikely to have affected the study outcomes, because no effect was seen on the number of oocytes collected or the total dose of FSH/hMG. Furthermore, this group had the most favorable outcome.

Although the rates of fertilization and cleavage were similar in both groups, there is growing evidence of a clear link between an increased proportion of high-quality day 3 embryos and likelihood of pregnancy, implantation, and blastocyst formation (34). Therefore, our observation of a significantly higher proportion of high-quality cleavage-stage embryos in insulin-supplemented medium (Table 2) may provide supporting evidence of a beneficial effect of early inclusion of insulin into culture medium.

Of clinical significance, this study's higher rate of embryo compaction on day 3 in the intervention group opens the door for further studies into whether insulin inclusion would improve implantation, given reports of a correlation between implantation and embryo compaction (20).

We found a significantly higher rate of blastocyst formation and improved blastocyst quality after insulin supplementation. Given the available evidence of a better outcome correlated with blastocyst transfer (35), and the higher rate of blastocyst cryopreservation in the treatment group, it may be that insulin supplementation would have a positive effect on cumulative pregnancy rate. It is, therefore, likely to be a long-term cost-effective treatment option for the

IVF community and an important factor in the development of a single-embryo transfer policy.

Our findings have provided a positive correlation between insulin supplementation of embryo culture media and the rates of clinical and ongoing pregnancies. The favorable effect of insulin on post-ICSI clinical outcomes was further confirmed by the significantly higher rate of twin pregnancies in the insulin-supplemented group. Therefore, insulin could be worth routine inclusion in culture medium, because the higher rates of clinical and ongoing pregnancies may result in a higher live birth rate. In addition, because the higher rate of twin pregnancy could suggest a positive impact of insulin on preimplantation embryo development leading to healthier intrauterine pregnancies, this would provide support for a single-embryo transfer policy.

Although insulin was shown in this study to be associated with a positive impact on early human embryo development in vitro, and on clinical and ongoing pregnancies, the exact mechanisms of a regulative role of insulin on human embryo development remain to be determined. The present study was not able to show a significant difference in implantation; this finding may be because the difference in implantation may need a narrower range of detection than the clinical pregnancy rate, such that a larger sample size of the number of transferred embryos may be required.

Although our results indicate that insulin represents a likely candidate for inclusion in human embryo culture, we were unable to rule out the possibility that the favorable outcomes were also regulated by additional mechanisms. Therefore, further studies to ascertain insulin's effect on human embryo development may be required.

In conclusion, the results of this comparative study show that early human embryo culture in insulin-supplemented single-step culture media from day 0 to day 5/6 significantly improved embryo development and clinical and ongoing pregnancy rates. This is expected to have direct implications for the strategies of improving human embryo culture media. However, whether our findings hold true for all media or for other patient populations is unknown. Therefore, a multicenter randomized controlled trial is needed to confirm our findings.

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