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Increased implantation rate after intrauterine infusion of a small volume of human chorionic gonadotropin at the time of embryo transfer: a randomized, double-blind controlled study

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Abstract

Purpose Intrauterine human chorionic gonadotropin (hCG) infusion at the time of embryo transfer (ET) has resulted in controversial results. We evaluated the effects of intrauterine infusion of a small volume of hCG at the time of ET in fresh and frozen–thawed cycles.

Methods Infertile women scheduled for ET with either fresh or frozen–thawed cycles were enrolled and randomized into two groups ($n = 100$ each): an hCG group, who received 500 IU of hCG in 10 µL culture medium infused into the uterine cavity using a soft catheter 4 min before ET; and a control group, who received 10 µL of culture medium alone by the same technique. The primary outcome was the implantation rate. The secondary outcomes were clinical pregnancy and live birth rate.

Results Two hundred infertile women aged 18–43 years, undergoing fresh or frozen–thawed ET were enrolled, regardless of any previous transfer cycles. The implantation rate was significantly higher in the hCG group compared with the control group (28.8% vs. 18.2%, $p = 0.030$). The clinical pregnancy rates were similar in both groups (42% vs. 30%, $p = 0.077$). The live birth rates were also similar (29% and 23% in the hCG and control group, respectively).

Conclusions Intrauterine infusion of a small volume of hCG at the time of ET can significantly improve the implantation rate, while the clinical pregnancy rate may only be improved in younger patients (aged < 40 years). This technique may thus be of benefit to patients undergoing clinical infertility treatment.

Keywords Intrauterine hCG · Implantation rate · Clinical pregnancy rate · Live birth rate · Embryo transfer

Introduction

The problem of infertility is on the rise worldwide, and was reported to affect up to 12% of the couples in Thailand [1]. Many methods and procedures have been invented to treat it. In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are the assisted reproductive techniques that have provided the highest successful pregnancy rates, reaching 50% in some studies [2]. However, there are still some patients who do not conceive. Variations in the pregnancy rate are associated with every aspect of assisted reproductive technology, such as the methods used for controlled ovarian hyperstimulation (COH), oocyte retrieval, IVF, embryo culture and embryo transfer (ET). The embryo implantation rate depends on a range of factors involving the endometrium, embryo quality and ET technique. The key point of ET is to put the 'best' quality embryo at the right position of the endometrial cavity exactly in the optimal implantation window, when the best endometrial receptivity is provided. Many techniques have been developed in clinical and laboratory settings; however, the pregnancy rate remains around 30% per cycle [3, 4]. Implantation failure is responsible for about 50–75% of the pregnancy losses [5, 6].

Implantation in the endometrium is an extremely complex, yet well-coordinated process. It consists of apposition, adhesion and invasion. The procedure is regulated by many factors, including ovarian hormones, cytokines, transcription factors, growth factors and extracellular matrix proteins [7]. Two early embryonic signals found are human chorionic

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gonadotropin (hCG) and interleukin (IL)-1 [8]. Of these, hCG is believed to be the most important [9]. It is a placental hormone needed to maintain a normal pregnancy. Blastocysts can produce hCG as early as 6–8 days after fertilization [10, 11]. A good embryo containing sufficient trophoblasts can produce enough hCG within an adequate doubling time (< 10 h) to assist embryo recognition in vivo [12]. Higher levels of secreted hCG are associated with higher implantation rates. Together with its free-β subunit, hCG facilitates trophoblastic invasion [13]. Enhanced invasion is correlated with raised levels of matrix metalloproteinase (MMP)-2, MMP-9 and the activity of urokinase-type plasminogen activator [13]. In an in vitro study, hCG also stimulated human peripheral blood mononuclear cells (PBMCs) which possess abundant hCG receptors; thus hCG might stimulate trophoblastic invasion through cytokines secreted by PBMCs [14]. The process of embryo implantation needs extensive angiogenesis at the maternal–fetal interface. Numerous cytokines and growth factors are produced by both the preimplantation stage embryo and the endometrial microenvironment.

Licht et al. investigated the direct effects of hCG on the human endometrium and found that the intrauterine administration of hCG (500 IU/mL) stimulated the production of leucocyte inhibitory factor (LIF), vascular endothelial growth factor (VEGF), and MMP-9, while the levels of intrauterine insulin-like growth factor binding protein (IGFBP)-1 and macrophage colony-stimulating factor (M-CSF) were inhibited. These results showed multiple direct effects of hCG on the endometrium during the time of implantation [15, 16]. Xiao-Yen et al. measured the hCG levels secreted from human embryos into the embryo culture medium. They concluded that there is a positive correlation between embryonic hCG production and implantation rate. Thus, secreted β-hCG from embryos could be a useful marker for embryo selection for ET in ICSI cycles, aiming for better implantation and pregnancy outcomes [17].

Some studies, including randomized controlled trials (RCTs), have investigated the benefits of intrauterine hCG in terms of pregnancy outcomes. The first study was conducted by Mansour et al. [18], proposing that the effective dose of intrauterine hCG that could improve pregnancy outcomes should be 500 IU, but not 100 or 200 IU. After 2011, all studies on intrauterine hCG used 500 IU dosing [19–21]. Only one study from Zarei et al. changed to the use of recombinant hCG (rhCG) at 250 μg instead of urinary hCG at 500 IU, and had good and consistent outcomes [22]. Although there have been many different details between studies such as the intrauterine hCG infusion volume (20–50 μL) and the lag time between hCG infusion and ET (less than 3 min to 12 min), most RCTs have reported significant positive effects of intrauterine hCG on pregnancy outcomes, except for that by Hong et al. In that study, the sustained implantation and ongoing pregnancy rates of blastocyst-stage ET were found to be improved after an endometrial infusion of hCG, but with no statistical significance [20]. The technique is safe and no adverse effects have been reported. One meta-analysis [23] confirmed that women undergoing infertility treatment with IVF or ICSI might benefit from intrauterine hCG infusion before ET by significantly increasing clinical and ongoing pregnancy rates. However, the meta-analysis reported by Osman et al. did not find any advantage of hCG infusion in terms of live birth rate or the spontaneous abortion rate [24]. Therefore, further studies are needed.

Most studies infused 20–50 μL of medium at the first catheter insertion and ET was performed after a short lag time. Usually, only 20 μL of medium is infused in routine ET. Therefore, a higher volume of liquid placed in the uterine cavity might displace or even expel the embryo from the original position [25, 26]. On the other hand, a volume less than 10 μL has a negative effect as well [27]. Therefore, reducing the medium volume or hCG solution volume before ET might facilitate correct embryo placement. Our hypothesis was that if a small volume of concentrated hCG was to be infused inside the uterine cavity shortly before ET, it would stimulate the production of cytokines associated with embryo adhesion and implantation, and help in enhancing implantation and improve pregnancy outcomes in ICSI cycles. Therefore, the primary objective of the study was to evaluate the effects of intrauterine infusion of 10 μL hCG shortly before ET on the clinical pregnancy rate in both fresh and frozen–thawed ET cycles. The secondary outcomes were to determine the effects of intrauterine hCG infusion on implantation and chemical pregnancy rates.

Materials and methods

This study was a randomized double-blind controlled study, conducted at an infertility unit of a university hospital. The study protocol was approved by the hospital's Institutional Review Board (Si 701/2015) and has been summited to clinical trials.gov: ID number NCT02668965. Participants were enrolled by a research nurse from December 2015 to March 2016 after the informed consents were granted. Infertile women, aged 18–43 years who attended the infertility unit and scheduled for ET in both fresh and frozen–thawed cycles were included in this study, regardless of any previous transfer cycles. Exclusion criteria were an azoospermic male partner, no oocytes retrieved, no embryos available for transfer, failure to have the proper endometrial thickness for ET (< 8 mm measured by transvaginal ultrasound), the use of donor oocytes, or in vitro oocyte maturation cycles.

Study protocols

Routine laboratory investigations included a complete blood count, hemoglobin typing, measures of Hepatitis B S antigen, laboratory tests for venereal diseases, antibodies to human immunodeficiency virus, and the levels of follicle stimulation hormone (FSH), luteinizing hormone, estradiol, and prolactin in female patients, and FSH and testosterone in male patients. Semen analyses for the male partners were carried out according to the WHO 2010 manual [28] and completed before starting the treatment. All the laboratory results, complete history evaluation and physical examination records were collected as the baseline characteristics. After obtaining informed consent, all the enrolled participants proceeded to standard management in a COH cycle or for endometrial preparation in a frozen–thawed ET cycle.

COH with fresh ET

The COH protocol and the initial dose of gonadotropins (Gonal-F®, Merck Serono, Italy) were chosen according to the patient's characteristics. Patients were monitored by transvaginal ultrasound (Aloka Prosound Alpha 7, Hitachi Aloka Medical America, Inc., Twinsburg, OH, USA) every 2–4 days until there were at least 1 or 2 follicles with a mean diameter ≥ 17 mm. The gonadotropin dose was adjusted during stimulation depending on the patient's individual ovarian response. Either 250 μg of recombinant hCG (Ovidrel®, Organon, Oss, The Netherlands) or 5000–10,000 IU of urinary hCG (Pregnyl®, Organon) was administered for ovulation induction. Oocyte retrieval was performed 34–36 h later. All oocytes were inseminated using ICSI. Patients who had no oocytes retrieved or embryo available for transfer were excluded from the study.

Endometrial preparation for frozen–thawed ET

For artificial endometrial preparation, oral estradiol hemihydrate (Estrofem®, Novo Nordisk A/S, Denmark) was commenced on the second day of the menstrual cycle at a dosage of 4 mg/day. Endometrial thickness was measured by transvaginal ultrasound until an adequate endometrial thickness had been achieved. Endometrial thickness ≥ 8 mm with a triple-layer appearance was deemed acceptable for ET. Both transvaginal progesterone (8% Crinone®, Merck Serono Ltd., Feltham, UK) and injected progestin (Proluton Depot®, Bayer HealthCare Pharma., Bangkok, Thailand) were used in this study and were applied 3–5 days before ET to synchronize the endometrium with the age of the transferred frozen–thawed embryo(s).

Embryo transfer

On the day of ET, the patients awaiting either fresh or frozen–thawed ET cycles were allocated randomly into two groups using block-of-10 computerized randomization numbers allocated at a ratio of 1:1 by the research nurse and sealed in individual envelopes. All the participants were assigned according to the randomization disclosed by the embryologist. The embryologist prepared the medium or hCG solution according to the allocation instructions enclosed in a blank envelope. Patients and their physicians were blinded throughout the procedure. Embryos were transferred after either 3 or 5 days of culture. The infusion mixture was prepared by dissolving 1500 IU of purified urinary hCG powder (Pregnyl®, Organon) in 30 μL of embryo culture media (Sydney IVF Blastocyst Medium or Sydney IVF Cleavage Medium, Cook®, Bloomington, IN, USA). The final preparation used for each patient was 500 IU hCG in 10 μL.

During ET, the patient was put in a lithotomy position. After a speculum had been inserted, the cervical mucus and any vaginal discharge were removed with small sterile cotton balls. In the hCG group, 500 IU of hCG in 10 μL culture medium was infused into the uterine cavity using a soft catheter, while in the control group, 10 μL of culture medium was infused via the same technique. One to three embryos of the best quality available were transferred by another catheter 4 min later. The process was completed after the embryologist confirmed that no embryos retained in the catheter. After ET, all patients received luteal phase support with transvaginal micronized progesterone (8% Crinone®, Merck Serono Ltd., Feltham, UK) and injected progestin (Proluton Depot®, Bayer HealthCare Pharma., Bangkok, Thailand).

Outcome measurements

Serum β-hCG levels were measured around 2 weeks after ET using an immunology analyzer (Cobas e601, Roche Diagnostics, Basel, Switzerland). If the serum β-hCG was positive, transvaginal ultrasonography (Aloka Prosound Alpha 7) was performed 2 weeks later to identify the number and location of gestational sac(s), and to evaluate any fetal heartbeat. The implantation rate was defined as the number of gestational sac(s) seen from transvaginal ultrasound based on the number of transferred embryos. Clinical pregnancy was defined as evidence of pregnancy by clinical or ultrasound parameters (ultrasound visualization of a gestational sac, embryonic pole with a heartbeat). Multiple gestational sacs in any one patient were counted as one clinical pregnancy. Live birth was defined as live born after 20 weeks gestation. All the live births were confirmed by hospital

records and telephone calls. Patients who lost contact were interpreted as abortion.

Sample size and statistical analysis

The sample size was calculated based on the study of Santibanez et al. 2014 [21] which included participants having similar characteristics. The study showed that the clinical pregnancy rate was improved from 33% in the control group to 50.4% in the hCG group. We determined a 1-sided alpha error at 0.05 (type I error = 5%, 1-sided) and power of the test = 80% (type II error = 20%). The required sample size was calculated using the formula:

$$n = \left(\frac{Z_{\alpha/2}\sqrt{2p(1-p)} + Z_{\beta}\sqrt{p_1(1-p_1)+p_2(1-p_2)}}{(p_1 - p_2)} \right)^2$$

where n is the sample size; α is the type I error = 5%, 1-sided ($Z_{\alpha/2} = 1.645$); β is the type II error = 20% ($Z_{\beta} = 0.84$); p_1 is the clinical pregnancy rate in control group = 0.33; p_2 is the clinical pregnancy rate in study group = 0.504; and $p = (p_1 + p_2)/2 = (0.33 + 0.504)/2 = 0.417$.

The sample size calculation showed that we needed 100 subjects per group. Data were analyzed using PASW Statistics version 18.0 (SPSS Inc., Chicago, IL, USA). Demographic and characteristic data were characterized using the mean and standard deviation (SD) when normally distributed, otherwise as the median plus range. Numbers and percentages are reported for categorical data. Characteristics between groups were compared using unpaired Student's t tests for continuous data with a normal distribution, and the Mann–Whitney nonparametric U test for continuous data with a non-normal distribution. Categorical data were compared by Chi-squared tests. All outcome rates were compared using Chi-squared tests and are reported as relative risk (RR) with 95% confidence interval (CI). A p value of < 0.05 was considered statistically significant.

Results

A total of 291 patients were assessed for eligibility. Thirty-four patients did not meet the inclusion criteria and 57 had an ET cancellation because of one or more of the exclusion criteria (Fig. 1). Finally, 200 patients were enrolled and randomized to the study. There were 29 and 23 live births confirmed in the hCG and the control groups, respectively. Seven cases in the hCG group and four cases in the control group lost contact for live birth report. The baseline characteristics of the patients were similar between the two groups (Table 1). There were no significant differences in age, body mass index, basal FSH level, type of infertility or the COH protocol used. The numbers of oocytes retrieved, metaphase II oocytes, and the fertilization rates of both groups were similar. Both fresh and frozen–thawed ETs were included in this study, with frozen–thawed ET predominating in both groups. The mean number of embryos transferred was 1.91 ± 0.473 in the hCG group and 1.87 ± 0.597 in the control group. The quality of transferred embryos was also similar between two groups.

The clinical pregnancy rates were higher in the hCG group compared with the control group; however, this was not statistically significant. Nevertheless, the implantation rate was increased in the hCG group ($p = 0.030$) and had an RR of 1.58 (95% CI 1.09–2.31; Table 2). Embryo quality tends to decrease with the age of the mother, resulting in lower implantation and ongoing pregnancy rates, especially in women aged > 40 years [29]. To analyze the difference in patient response between expected good and poor-quality embryos, a subgroup analysis was performed. The clinical pregnancy rate was significantly higher in the hCG group than in the control group among patients aged < 40 years (45.6% vs. 27.8%; $p = 0.021$) but differences between groups remained insignificant in patients aged ≥ 40 years. There is different hormonal milieu in fresh and frozen cycles. Therefore, another subgroup analysis was performed. The clinical pregnancy rate in fresh embryo transfer cycle was higher in the hCG group (51.4%) than in the control group (33.3%), however, did not reach the statistical significance. In the frozen cycle, the clinical pregnancy rates were comparable (36.5% vs 28.8%) in the hCG and control group, respectively. The confirmed live births were similar in both subgroups as well (Table 2).

Two cases of blighted ova were identified in this study: one in each group. There was a much higher twin pregnancy rate in the hCG group compared with the control group (28.6% vs. 10%; $p = 0.035$). All the twin pregnancies in the hCG group occurred from double ET with 100% implantation rate. Eight of them conceived from day 5 transfers and four from day 3 transfers. No triplets or higher order multiple pregnancies were found.

Discussion

In this RCT, we demonstrated the benefit of intrauterine infusion of a small volume of culture medium containing 500 IU hCG at the time of ET. The implantation rate increased significantly in the hCG group, and there were trends for improvements in the clinical pregnancy rates. The clinical pregnancy rate was 30% in the control group compared with 42% in the hCG group with an RR of 1.40 (95% CI 0.96–2.04). This result is consistent with other reports [18–22]. Since the study population, patient ages and transfer cycles were different; therefore, the difference

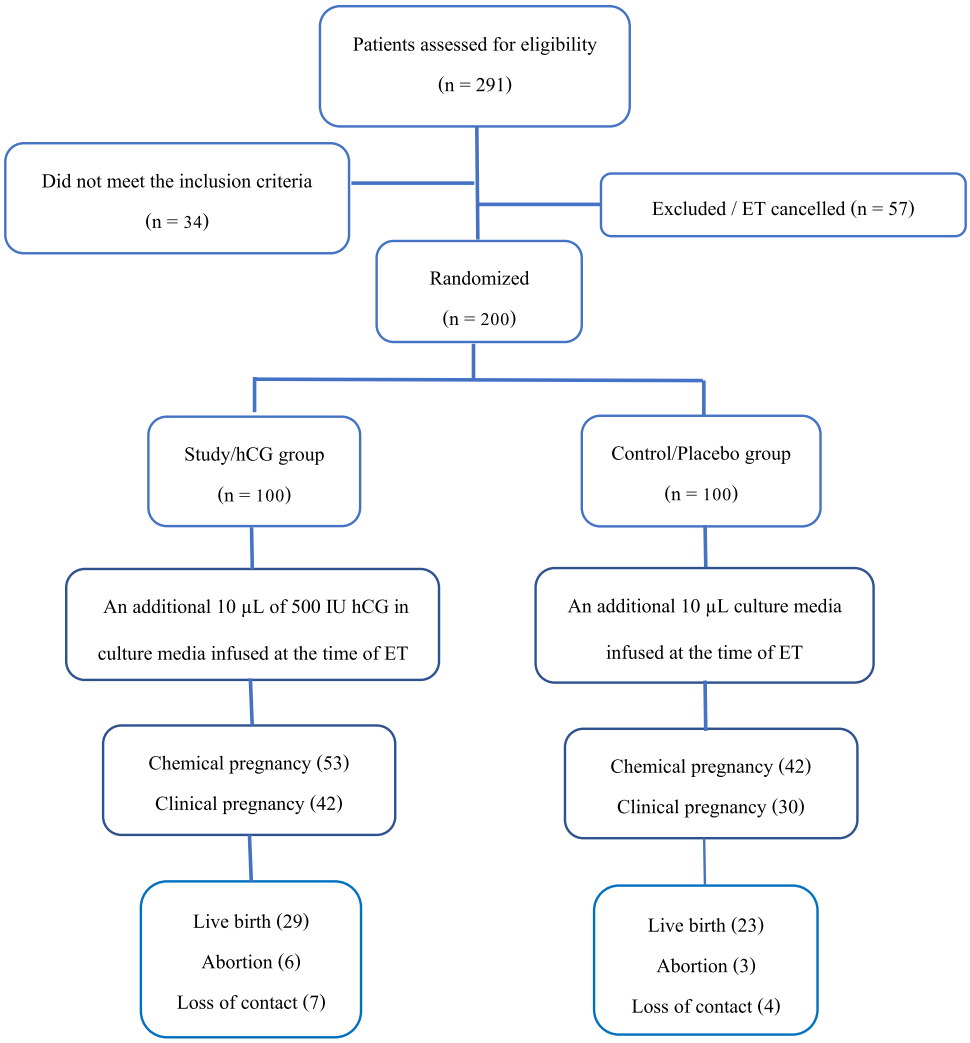

Fig. 1 Flow diagram of participants throughout the study. A total of 291 patients were assessed for eligibility. Thirty-four patients did not meet the inclusion criteria and 57 experienced ET cancellation due to one or more of the exclusion criteria. Two hundred patients were enrolled and randomized to the study. One hundred patients were allocated in each group. There were 53 and 42 pregnancies occurring in the hCG and control group, respectively. No drop out cases

of pregnancy rate was not as high as the report of Santibañez et al. Additionally, intrauterine hCG infusion with an improvement in the implantation rate was associated with a higher rate of twin pregnancy (12 vs. 3 sets of twins). All the twin pregnancy in the hCG group occurred from two embryos being transferred at ET with 100% implantation rate. This suggests that intrauterine hCG infusion would be appropriate for couples with only one embryo to increase the implantation rate and reduce the risk of multiple pregnancies. Our subgroup analysis also found a trend for increased implantation rates in the hCG group for both day 3 and day 5 ETs; however this was not statistically significant. This was consistent with previous studies that transferred only cleavage-stage embryos, showing positive results following an hCG infusion [18, 21, 22]. In contrast, studies that used blastocyst transfer did not support the efficacy of hCG infusion prior to ET [20, 30]. This discrepancy might have arisen from the different stage of embryo development. Blastocysts have already started to secrete hCG [11], so the additional hCG at ET was probably unnecessary or insufficient to improve outcomes significantly. In the subgroup analysis of fresh cycles, the clinical pregnancy rate in the hCG group was 1.54 times that in the control group. Although this discrepancy was clinically significant, it is not statistically significant. It may due to the low sample size in the subgroup analysis. Nevertheless, this finding suggested the possible benefit of hCG infusion in the fresh cycles to rescue the disturbed endometrial receptivity caused by supraphysiologic levels of estrogen and progesterone. Anyway, the molecular basis of the theory should be discovered in future studies.

The hormone hCG has numerous functions. It promotes progesterone production, promotes angiogenesis in uterine vasculature, forestalls any immune or macrophage action from the mother on invading placental tissue, enhances smooth muscle cell proliferation, suppresses myometrial contractions during pregnancy by regulating gap junctions, and also signals the endometrium about forthcoming embryo implantation [31, 32]. The role of hCG during implantation in regulating immunological reactions and angiogenesis at the embryo–maternal interface has been

Table 1 Baseline characteristics

	hCG group ($n=100$)	Control group ($n=100$)	p value[#]
Age (years)	36.97 ± 3.16	36.68 ± 3.21	0.521
BMI (kg/m^2)	21.72 ± 3.16	22.00 ± 2.87	0.505
Infertility type			0.755
Primary/secondary	70/30	72/28	
Basal FSH (IU/ml)	7.67 ± 2.41	7.43 ± 2.53	0.509
COH Protocol			0.539
Antagonist	85	79	
Agonist	11	15	
Mild stimulation	4	6	
No. of oocyte retrieved	12.55 ± 7.62	12.54 ± 7.79	0.993
No. of MII oocytes	9.95 ± 6.03	9.85 ± 5.90	0.906
No. of fertilized embryos	7.05 ± 4.77	6.57 ± 4.11	0.447
Embryo transfer			
Fresh/frozen	37/63	27/73	0.130
Days of transferred embryo(s)			0.748
Day 3	40	36	
Day 4	5	7	
Day 5	55	57	
No. of transferred embryo(s)			0.096
1 embryo	16	25	
2 embryos	77	63	
3 embryos	7	12	
Quality of transferred embryo(s)			0.967
Good	56	56	
Moderate	35	34	
Poor	9	10	

The baseline characteristics of the patients were comparable between the two groups. There was no difference in age, body mass index (BMI), basal follicle stimulating hormone (FSH) level, type of infertility and the controlled ovarian hyperstimulation (COH) protocol used. The number of oocytes retrieved, metaphase II oocytes, and the fertilized embryos of both groups were similar, too. Both fresh ETs and frozen ETs were included in this study; however, frozen ET was predominated in both groups. The mean transferred embryos were 1.91 ± 0.473 in the hCG group and 1.87 ± 0.597 in the control group. The quality of transferred embryo(s) was also comparable between two groups, containing 56% good quality embryos in both groups

Values represent mean ± SD or n (%)

[#]Chi-Square test or unpaired T test

Table 2 Pregnancy outcomes

	hCG group	Control group	Relative risk (95% CI)	p value[#]
Clinical pregnancies	42/100	30/100	1.40 (0.96–2.04)	0.077
Implantation rate	55/191 (28.80%)	34/187 (18.18%)	1.58 (1.09–2.31)	0.030[*]
Live birth rate	29/100 (29%)	23/100 (23%)	1.26 (0.79–2.02)	0.33

The clinical pregnancy rate was higher in the hCG group compared with the control group (42% vs. 30%, respectively), however, not statistically significant. Nevertheless, the implantation rate was increased in the hCG group ($p=0.030$)

[#]Chi-square test

[*]Statistical significance

described in detail [9, 33–36], and a positive association between early embryo hCG secretion and implantation rate has been confirmed by many studies [10, 11, 15, 34, 37–39]. Licht et al. reviewed the main functions of intrauterine hCG and concluded that it is involved in modulating endometrial receptivity at four important levels [15]. First, hCG inhibits the production of IGFBP-1, resulting in an increased level of IGF-II and angiogenesis that can lead to prolongation of the implantation 'window'. Second, hCG increases the local production of VEGF, which is a proangiogenic growth factor, so angiogenesis in the endometrium at the implantation site is increased. Third, hCG stimulates the production of LIF, a cytokine required for implantation, while it inhibits the production of M-CSF at the same time: both are necessary for the implantation process involving embryo–maternal crosstalk. Fourth, hCG inhibits the levels of tissue inhibitors of matrix metalloproteinases and MMP-9, enabling the embryo to modulate the process of endometrial tissue remodeling. All these effects act together to enable stable implantation, showing that hCG is an important signal during implantation and in the stabilization of pregnancy.

In this study, only a small volume of concentrated hCG solution was used. The uterine cavity can store only a small volume of fluid. Large volume of fluid infused into the uterus before ET might displace or even expel the embryos from the intended implantation site and reduce the implantation rate [25, 26]. However, in our study, the implantation and pregnancy rates were not higher than those reported using higher volumes of hCG. In any case, a low volume is better in terms of shorter lag time needed before ET to avoid the risk of embryos being expelled [18, 19, 22].

This study provides the first report of live birth rate post-intrauterine infusion of hCG in both fresh and frozen embryo transfer cycles. However, there were still some limitations. The COH protocols varied between the groups. Both fresh and frozen–thawed ET cycles were included. There are some differences between such cycles. For example, in the fresh ET cycles, an injection of hCG had already been given to induce oocyte maturation. This might distribute to the endometrium, causing some changes during the implantation window. Therefore, the clinical implications should be considered individually. Further, studies including only fresh or frozen–thawed ET cycles should be considered.

All previous reports on this technique used whole hCG molecule, which is not the main element in the secretome of cultured embryos. Hyperglycosylated hCG and the free-β subunit of hCG are the main components secreted by embryos cultured in vitro, and are suggested to be potential markers for infertility management [30]. Accordingly, studies using specific forms of hCG are warranted to be performed in the future, especially in cases of blastocyst transfer.

Conclusion

Intrauterine infusions of a small volume of hCG at the time of ET significantly promoted the implantation rate. The clinical pregnancy rate was improved, but only in patients aged < 40 years. This technique may thus be of benefit to patients undergoing clinical infertility treatment.

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Author contributions PL: project development, and manuscript editing. IT: data collection and analysis, manuscript writing and editing. SB: project development, data collection, data analysis, and manuscript writing. SP: manuscript editing. JP: manuscript editing. RC: manuscript editing.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Hospital Institutional Review Board, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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