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GYNECOLOGIC ENDOCRINOLOGY AND REPRODUCTIVE MEDICINE

Intrauterine administration of human chorionic gonadotropin improves the live birth rates of patients with repeated implantation failure in frozen-thawed blastocyst transfer cycles by increasing the percentage of peripheral regulatory T cells

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Abstract

Introduction Repeated implantation failure (RIF) frustrates both patients and their clinicians. Our aim was to observe the effects of intrauterine administration of human chorionic gonadotropin (hCG) on pregnancy outcomes of patients who received frozen-thawed embryo transfer (FET).

Methods A prospective cohort study was conducted to evaluate the impact of intrauterine administration of hCG on pregnancy outcomes in FET cycles of patients with RIF from January 1st 2016 to December 31st 2016. The treatment group ($n = 153$, 152 cycles) received an infusion of 500 IU of hCG diluted in normal saline 3 days before embryo transfer. The control group ($n = 152$, 151 cycles) received embryo transfer with a previous intrauterine injection of normal saline without hCG. Early morning fasting blood samples were obtained from each patient for the measurement of peripheral regulatory T cells (Tregs) on the day of embryo transfer. The outcome parameters including Tregs in each group were compared.

Results The patients in the hCG-treated group had significantly higher clinical pregnancy rates, implantation rates and live birth rates than the controls (37.5% versus 25.17%, 29.19% versus 19.4%, 26.97% versus 17.22%, respectively). They also had significantly higher percentages of peripheral Tregs than the controls ($6.1 \pm 0.6\%$ versus $5.4 \pm 1.0\%$). In addition, the clinical pregnancy rate, implantation rate and live birth rate in patients who received blastocyst transfer were significantly higher in the hCG-treated group when compared to the control group (41.38% versus 26.44%, 42.22% versus 26.14%, 33.33% versus 17.24%, respectively). We also showed that the clinical pregnancy rate, implantation rate and live birth rate were significantly higher in hCG-treated group when compared to the control group (49.12% versus 28.07%, 49.15% versus 28.07%, 40.35% versus 17.54%, respectively) of RIF patients with blastocyst transfer under 35 years, while there was on difference in patients above 35 years.

Conclusions Intrauterine administration of hCG significantly improves the clinical pregnancy rate, implantation rate and live birth rate in FET cycles of patients with RIF by increasing Tregs. The treatment improves the pregnancy outcomes much more for younger RIF patients transferred blastocysts.

Keywords Human chorionic gonadotropin · Tregs · Embryo transfer · Live birth rate

Introduction

Successful implantation is a complex process involving the receptive endometrium and a high quality embryo. Any problem stemming from these two main players can adversely affect the cross-talk between the embryo and the endometrium, which can result in implantation failure. When transferred embryos of good quality fail to implant after several in vitro fertilization (IVF) treatment cycles, RIF

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is determined. RIF remains a variable definition, but the definition of failure of implantation after three or more embryo transfers or transfer of ten or more high-quality embryos has been taken as criteria by many researchers [1]. The causes of implantation failure include maternal factors and embryonic causes [2]. However, for many of patients with RIF the cause cannot be identified, which creates frustration both patients and their clinicians.

It is well known that the progression of pregnancy requires immunological tolerance at the maternal–fetal interface. A cascade of cytokines mediates this dialogue and is important in the cross-talk between the immune and endocrine systems. hCG is the first known human embryo-derived signal which influences immunological tolerance and angiogenesis at the maternal–fetal interface. It, as the earliest embryonic product, could be a key regulator in triggering the complex process of embryo implantation. In addition, hCG can prolong endometrial receptivity, support appropriate endometrial angiogenesis, and modulate endometrial tissue remodelling to promote embryo implantation [3–5].

The hCG-subunits are already secreted by two-cell stage embryos and increase to high concentrations at the blastocyst stage [6]. Therefore, hCG already activates the embryo-endometrial dialogue before the embryo arrives in the uterine cavity on day 5 and 6 as a blastocyst. It may function as a modulator of embryo-maternal communication and maternal immune response during implantation [7, 8]. However, IVF patients lack the effect of hCG on the uterus prior to embryo transfer, especially blastocyst transfer. IVF decreases hCG signalling to the endometrium during the early days of the embryo development, which could contribute to the relatively low implantation rate [9, 10]. Therefore, there have been many recent studies where researchers have attempted to inject hCG into uterine cavity before embryo transfer and conclude that this treatment significantly improves embryo implantation and clinical pregnancy rates [11–14]. However, others show no beneficial effect before blastocyst transfer [10, 15]. This could be because there is not enough time for hCG to have any significant beneficial effect on the endometrium before implantation [3, 9].

On the other hand, the percentages of Tregs are significantly lower in patients with RIF [16]. Tregs are necessary for the maintenance of maternal–fetal tolerance. hCG increases Tregs in periphery and attracts Tregs to the maternal–fetal interface during pregnancy [4]. Moreover, a recent study has showed that hCG could upregulate Tregs [16]. Based on these findings, we hypothesized that if we inject hCG into the uterine cavity before embryo transfer for RIF patients, it may improve embryo implantation by increasing Tregs. However, few studies conducted to date have examined Tregs in peripheral blood of patients with RIF after injecting hCG. So, this study aimed to determine whether intrauterine administration of hCG improved pregnancy outcomes by increasing Tregs in RIF patients who received FET.

Materials and methods

Subjects

A total of 305 RIF patients who had an indication for a frozen-thawed embryo transfer cycle were enrolled in the study from January 1st 2016 to December 31st 2016. Each patient gave written consent at the time of treatment for the future use of their clinical data. At our center, RIF is defined as implantation failure after three or more transfers of high-quality embryos.

Ethics

This study was a prospective cohort study that was approved by the Ethical Committee of Yantai Yuhuangding Hospital. This study was performed according to the ethical guidelines regarding studies in which human gametes or embryos are used as materials. These guidelines are issued by the Ethics Committee of the China Society of Obstetrics and Gynecology.

Inclusion criteria were as follows: a history of RIF, age ≤ 45 years, BMI 19–30 kg/m^2, basal FSH < 10 IU/L, normal uterine cavity as assessed through hysteroscopy, and normal maternal and paternal karyotypes. Exclusion criteria were the following: severe uterine malformation, severe uterine adhesions, chromosomal abnormality, antiphospholipid syndrome, hydrosalpinx (if not surgically removed or ligated), any contraindication to pregnancy, thyroid or adrenal dysfunction, neoplasia, severe impairment of renal or hepatic function, and use of medications that might interfere with study evaluations (e.g., hormonal medication, prostaglandin inhibitors, psychotropic agents).

Study design

All enrolled patients were thoroughly informed about the novelty and unknown efficacy of intrauterine injection hCG in improving pregnancy outcomes. All patients were not performed preimplantation genetic screening. Each patient was included with only one treatment cycle. The patients were randomized into two groups using a computerized random digit generator based on their registration number. In the treatment group ($n = 153$, 153 cycles), 500 IU of hCG was injected into the uterine cavity 3 days before embryo transfer. The patients in the control group ($n = 152$, 152 cycles) received an intrauterine injection of saline without hCG before embryo transfer. Maternal peripheral blood samples

were collected from each patient in sterile heparinized tubes on the day of embryo transfer for the measurement of Tregs. The outcome parameters were compared between the two groups.

Procedure

In this study, the regimens of endometrial preparation include natural and artificial cycles. Natural cycles were used for patients with spontaneous ovulatory cycles, and hormone replacement therapy (HRT) was used for patients with anovulatory cycles or no residual ovarian function.

Natural cycle

Transvaginal ultrasound monitoring was performed from the 10th day of the patients' menstrual cycle. When the mean diameter of the dominant follicle reached 14 mm, urinary luteinizing hormone (LH) was used to determine the timing of ovulation. A minimal endometrial thickness of 6 mm was required to perform FET. FET was scheduled 4 or 6 days after detecting ovulation according to the frozen embryo stage. Day 3 embryos were transferred 4 days after ovulation, and day 5 or day 6 embryos were transferred 6 days after ovulation. Luteal phase support was started from ovulation by administering micronized vaginal progesterone at a dose of 200 mg/12 h daily (Utrogest™ 200, Besins-Iscovesco, France) until 10 weeks of pregnancy.

Artificial cycle

The HRT for endometrial preparation was initiated with oral estradiol valerate on the 3rd day of menstrual cycle after a transvaginal ultrasonography. This protocol started with 2 mg/day on days 1–4, 4 mg/day on days 5–8, and 6 mg/day from day 9 onward. A second transvaginal ultrasonography was performed after 12–15 days of estrogen treatment. If endometrial thickness was more than 8 mm (at least 6 mm), the micronized vaginal progesterone was administrated at a dose of 600 mg/day (200 mg three times a day) (Utrogest™ 200, Besins-Iscovesco, France), and FET was scheduled. Day 3 embryos were transferred 4 days after administrating progesterone, and day 5 or day 6 embryos were transferred 6 days after administrating progesterone. Progesterone supplementation continued until 10 weeks of pregnancy or 14 days after embryo transfer if not pregnant.

In the study group, 500 IU of hCG was injected into the uterine cavity 3 days before embryo transfer. The hCG for intrauterine injection was prepared by adding 0.2 ml of normal saline to one vial containing 2000 IU of hCG (Choragon, Livzon Pharmaceutical Group, Inc., China). Then 50 µl of this solution containing 500 IU hCG was injected into the uterine cavity using an intrauterine insemination catheter (K-Soft-5000 catheter; Cook Medical, Inc., USA). Three days after the hCG injection, the embryos were then transferred.

All intrauterine injection and transfer procedures were performed by the same physician to avoid inter-operator variability. The embryologist was blinded to the medication assignment. Serumβ-hCG assay was measured on the 14th day after embryo transfer. In case of pregnancy, a transvaginal ultrasound was performed after 4 weeks from the embryo transfer and repeated as required. Clinical pregnancy was confirmed if the fetal heartbeat was observed by transvaginal ultrasound.

Flow cytometry

100 µl of fresh EDTA-K2 anticoagulated whole blood were mixed with PercPcy5.5-conjugated anti-human CD3, phycoerythrin (PE)-conjugated anti-human CD4, fluorescein isothiocyanate (FITC)-conjugated anti-human CD25 and Alexa Fluor 647-conjugated antihuman CD127 (BD Bioscience, San Jose, CA, USA). The reaction mixture was gently stirred and incubated for 30 min in the dark. Red blood cells were lysed with lysing solution (BD Biosciences) for 10 min, and washed with phosphate-buffered saline (PBS) followed by dilution in 0.5 mL of PBS for flow cytometric analysis. The flow cytometric analysis was performed using BD FACSCanto.

Statistical analysis

The clinical pregnancy rate was considered as the primary outcome for this study, with the live birth rate, implantation rate, miscarriage rate, multiple pregnancy rate, ectopic pregnancy rate, and percentage of Tregs assumed as correlated parameters of the primary outcomes. Secondary outcomes were other parameters, such as endometrial thickness, number of embryos transferred before this study, number of embryos transferred, number of high quality embryos transferred, endometrial preparation protocols (EPP), and embryo type.

Before this study, we have analyzed the clinical pregnancy rate of RIF patients undergoing FET in our center, and we found the rate was approximately 23.5%. After intrauterine administration of hCG before FET, the clinical pregnancy rate was approximately 37%. Considering these rates, and with $\alpha = 0.05$ and $\beta = 20\%$, a sample size of 143 patients in each group was calculated.

The data was analyzed by use of the SPSS-12.0 software. The continuous variables were presented as mean ± SD, and the proportions were presented as percentages. Differences between groups of continuous variables were analyzed with *t* test and the Chi-square test was used to assess differences

in proportions. $P < 0.05$ was considered to be statistically significant.

Results

305 patients were enrolled in the study. Of the 305 women, two cancelled embryo transfer (one experienced fever and one experienced diarrhoea) and were, therefore, excluded from the statistical analysis. 303 patients were analyzed in the present investigation. The hCG-treated group and control group consisted of 152 and 151 RIF patients, respectively. In this study, the hCG treatment was very safe and well tolerated by all patients. None of the patients experienced complications such as infection and vaginal bleeding. All of the patients' characteristics were comparable between the two groups, as no significant differences were observed between the two groups in terms of age, BMI, EPP, endometrial thickness, number of embryos transferred before the study, number of embryo transfer, number of high quality embryos transferred and embryo type (Table 1).

The outcomes of the hCG-treated and control groups are summarized in Table 2. The results showed the clinical pregnancy rate, implantation rate and live birth rate were statistically higher in the hCG-treated group than the control group (37.5% versus 25.17%, 29.19% versus 19.4%, 26.97% versus 17.22%). However, there were no differences in abortion rate (22.81% versus 26.32%), multiple pregnancy rate (8.77% versus 7.89%), or ectopic pregnancy rate (3.51% versus 2.63%), respectively.

In this study, Tregs were defined as $CD4^+CD25^+CD127^{dim/-}$ lymphocytes. As shown in Fig. 1, hCG-treated patients had a significantly higher percentage of $CD4^+CD25^+CD127^{dim/-}$Tregs in $CD4^+$ T cells than non-hCG-treated patients on the day of embryo transfer ($6.1 \pm 0.6\%$ versus $5.4 \pm 1.0\%$, $P < 0.05$).

In addition, we have conducted a sub-analysis of the cleavage stage embryos and blastocysts to see whether there is any difference in intrauterine injection hCG. The results showed the clinical characteristics of participants were similar in the hCG-treated and the control group either for the cleavage stage embryos or the blastocysts. Of the RIF patients who transferred cleavage stage embryos, the clinical pregnancy rate and implantation rate was higher in the hCG-treated group when compared to the control group (32.31%

Table 1 Patient characteristics in the hCG-treated and control groups

Parameter	hCG group	Control group	*P* value
Number of cycles	152	151	
Age (years)	34.83 ± 4.31	35.25 ± 4.94	0.43
BMI (kg/m^2)	23.63 ± 3.05	24.12 ± 3.28	0.18
EPP (number)			
Natural cycle	31 (31/152)	34 (34/151)	0.65
Artificial cycle	121 (121/152)	117 (117/151)	0.65
Endometrial thickness (mm)	9.40 ± 2.04	9.12 ± 2.23	0.25
Number of ET before the study	6.22 ± 1.80	6.13 ± 1.42	0.62
Number of ET	1.38 ± 0.49	1.33 ± 0.47	0.43
Number of high quality embryos	0.97 ± 0.70	0.98 ± 0.62	0.86
Embryo type (number)			
Cleavage-stage	65 (65/152)	64 (64/151)	0.95
Blastocyst	87 (87/152)	87 (87/151)	0.95

Values are presented as mean ± SD or percentages

Table 2 Outcomes in the hCG-treated and control groups

Parameter	hCG group	Control group	*P* value
Number of cycles	152	151	
Clinical pregnancy rate (%)	37.5 (57/152)	25.17 (38/151)	0.02*
Implantation rate (%)	29.19 (61/209)	19.4 (39/201)	0.02*
Live birth rate (%)	26.97 (41/152)	17.22 (26/151)	0.04*
Abortion rate (%)	22.81 (13/57)	26.32 (10/38)	0.69
Multiple pregnancy rate (%)	8.77 (5/57)	7.89 (3/38)	0.88
Ectopic pregnancy rate (%)	3.51 (2/57)	2.63 (1/38)	0.81

Values are presented as percentages. Clinical pregnancy rate: total number of clinical pregnancy/total transferred cycles; Implantation rate: total number of gestational sac/total embryos transferred; Live birth rate: total number of live delivery/total transferred cycles; Abortion rate: number of abortion/total number of clinical pregnancy; Multiple pregnancy rate: number of multiple pregnancy/total number of clinical pregnancy; Ectopic pregnancy rate: number of ectopic pregnancy/total number of clinical pregnancy

*Values are significantly different between groups ($P < 0.05$)

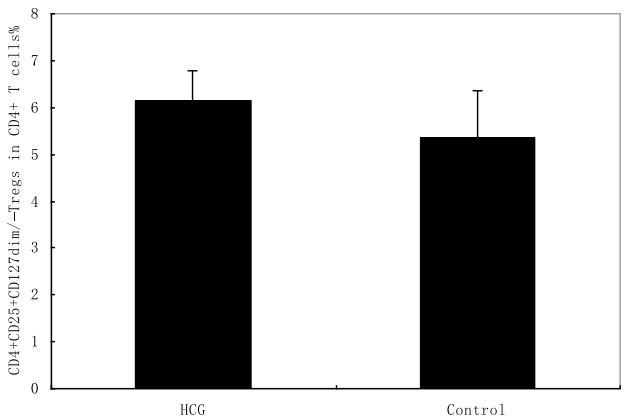

Fig. 1 The patients injected hCG into uterine cavity had a significantly higher percentage of $CD4^+CD25^+CD127^{dim/-}$Tregs in $CD4^+$ T cells than controls on the day of embryo transfer ($P < 0.05$)

versus 23.43%, 19.32% versus 14.16%), but this difference was not statistically significant. However, the clinical pregnancy rate, implantation rate and live birth rate were significantly higher in the hCG-treated group when compared to the control group (41.38% versus 26.44%, 42.22% versus 26.14%, 33.33% versus 17.24%, respectively) for the RIF patients who transferred blastocysts. The abortion rate in hCG-treated group was lower than that in the control group (16.67% versus 30.43%), although this was not statistically significant (Table 3). Therefore, the sub-analysis showed that intrauterine injection of hCG was better for the RIF patients who transferred blastocysts.

Moreover, we found the implantation rate was significantly higher in RIF patients who had blastocyst transfer when compared to RIF patients who transferred cleavage stage embryos (42.22% versus 19.32%, 26.14% versus 14.16%) in the hCG-treated group and control group, respectively. The live birth rate was also significantly higher in RIF patients who transferred blastocysts when compared with RIF patients who transferred cleavage stage embryos (33.33% versus 18.46%) in the hCG-treated group (Table 4). In our center, if the embryo is a blastocyst, then one embryo is transferred. If the embryo is a cleavage embryo, then two embryos are transferred. Therefore, the number of embryos transferred was significantly lower for patients who transferred blastocysts compared with patients who transferred cleavage stage embryos (1.03 ± 0.18 versus 1.83 ± 0.42, 1.01 ± 0.11 versus 1.76 ± 0.43) in both groups (Table 4).

In addition, we found that RIF patients who transferred blastocysts were significantly younger compared to those who transferred cleavage stage embryos (33.24 ± 3.44 versus 36.95 ± 4.45, 33.25 ± 3.96 versus 37.96 ± 4.87) in both groups, respectively (Table 4). This suggests that hCG treatment could improve pregnancy outcomes for blastocyst transfer, but does not have the same effect for cleavage stage transfer. Therefore, to observe whether the treatment is more effective for improving the pregnancy outcomes of younger RIF patients, we conducted a sub-analysis of RIF patients who transferred blastocysts above and below the age of 35. The results showed that the clinical pregnancy rate, implantation rate and live birth rate were significantly higher in hCG-treated group when compared with the control group (49.12% versus 28.07%, 49.15% versus 28.07%, 40.35% versus 17.54%, respectively) of RIF patients who transferred blastocyst under 35 years (Table 5). However, they were not statistically different between the two groups of RIF patients who transferred blastocyst above 35 years.

Table 3 The effects of intrauterine injection of hCG on the outcomes of RIF patients transferred blastocysts

Parameter	hCG group	Control group	*P* value
Number of cycles	87	87	
Clinical pregnancy rate (%)	41.38 (36/87)	26.44 (23/87)	0.04*
Implantation rate (%)	42.22 (38/90)	26.14 (23/88)	0.02*
Live birth rate (%)	33.33 (29/87)	17.24 (15/87)	0.01*
Abortion rate (%)	16.67 (6/36)	30.43 (7/23)	0.21
Multiple pregnancy rate (%)	11.11 (4/36)	8.69 (2/23)	0.76
Ectopic pregnancy rate (%)	0 (0/36)	0 (0/23)	–

Values are presented as percentages. Clinical pregnancy rate: total number of clinical pregnancy/total transferred cycles; Implantation rate: total number of gestational sac/total embryos transferred; Live birth rate: total number of live delivery/total transferred cycles; Abortion rate: number of abortion/total number of clinical pregnancy; Multiple pregnancy rate: number of multiple pregnancy/total number of clinical pregnancy; Ectopic pregnancy rate: number of ectopic pregnancy/total number of clinical pregnancy

*Values are significantly different between groups ($P < 0.05$)

Table 4 Outcomes of RIF patients transferred blastocysts or cleavage stage embryos in the hCG-treated and control group

Parameter	hCG cleavage	hCG blastocyst	*P* value	Control cleavage	Control blastocyst	*P* value
Number of cycles	65	87		64	87	
Age (years)	36.95 ± 4.45	33.24 ± 3.44	< 0.01*	37.96 ± 4.87	33.25 ± 3.96	< 0.01*
Number of ET	1.83 ± 0.42	1.03 ± 0.18	< 0.01*	1.76 ± 0.43	1.01 ± 0.11	< 0.01*
Clinical pregnancy rate (%)	32.31 (21/65)	41.38 (36/87)	0.25	23.43 (15/64)	26.44 (23/87)	0.68
Implantation rate (%)	19.32 (23/119)	42.22 (38/90)	< 0.01*	14.16 (16/113)	26.14 (23/88)	0.03*
Live birth rate (%)	18.46 (12/65)	33.33 (29/87)	0.04*	17.19 (11/64)	17.24 (15/87)	0.99
Abortion rate (%)	33.33 (7/21)	16.67 (6/36)	0.15	20 (3/15)	30.43 (7/23)	0.48
Multiple pregnancy rate (%)	4.76 (1/21)	11.11 (4/36)	0.41	6.67 (1/15)	8.69 (2/23)	0.82
Ectopic pregnancy rate (%)	9.52 (2/21)	0 (0/36)	–	6.67 (1/15)	0 (0/23)	–

Values are presented as mean ± SD or percentages. Clinical pregnancy rate: total number of clinical pregnancy/total transferred cycles; Implantation rate: total number of gestational sac/total embryos transferred; Live birth rate: total number of live delivery/total transferred cycles; Abortion rate: number of abortion/total number of clinical pregnancy; Multiple pregnancy rate: number of multiple pregnancy/total number of clinical pregnancy; Ectopic pregnancy rate: number of ectopic pregnancy/total number of clinical pregnancy

*Values are significantly different between groups ($P < 0.05$)

Table 5 The effects of intrauterine injection of hCG on the outcomes of RIF patients transferred blastocysts under 35 years

Parameter	hCG group	Control group	*P* value
Number of cycles	57	57	
Clinical pregnancy rate (%)	49.12 (28/57)	28.07 (16/57)	0.02*
Implantation rate (%)	49.15 (29/59)	28.07 (16/57)	0.02*
Live birth rate (%)	40.35 (23/57)	17.54 (10/57)	0.01*
Abortion rate (%)	14.29 (4/28)	31.25 (5/16)	0.18
Multiple pregnancy rate (%)	3.57 (1/28)	6.25 (1/16)	0.68
Ectopic pregnancy rate (%)	0 (0/28)	0 (0/16)	–

Values are presented as percentages. Clinical pregnancy rate: total number of clinical pregnancy/total transferred cycles; Implantation rate: total number of gestational sac/total embryos transferred; Live birth rate: total number of live delivery/total transferred cycles; Abortion rate: number of abortion/total number of clinical pregnancy; Multiple pregnancy rate: number of multiple pregnancy/total number of clinical pregnancy; Ectopic pregnancy rate: number of ectopic pregnancy/total number of clinical pregnancy

*Values are significantly different between groups ($P < 0.05$)

Discussion

The definition of RIF remains variable, but for the purpose of this study, we defined RIF as implantation failure after three or more transfers of high-quality embryos. Implantation failure is related to either maternal factors or embryonic causes. The "cross-talk" between the embryo and the endometrium is important for successful implantation. Being one of the earliest embryonic products, hCG is a prime embryo-endometrial signal and regulates the embryo-endometrial communication [3]. It has beneficial effect on the endometrial receptivity and induces gene expression cascade towards implantation [7, 9].

Several studies have shown that intrauterine injection of hCG before embryo transfer can significantly improve embryo implantation and clinical pregnancy rates in IVF [11–14]. However, this beneficial effect has not been demonstrated for intrauterine hCG administration before blastocyst transfer [10, 15]. It is thought that patients undergoing blastocyst transfer are more likely to lack of the effects of hCG on the uterus prior to embryo transfer when compared with those undergoing cleavage stage embryo transfer, as hCG probably has less time to have an effect on the endometrium [3, 15]. To overcome the limitations of the short duration of the intrauterine hCG effect before embryo transfer, we administered intrauterine hCG 3 days before embryo transfer in our study, using the same timing of the intrauterine hCG injection as the Huang et al. and Navali et al. [17, 18].

In our study, we show that intrauterine administration of hCG may significantly improve pregnancy rate, implantation rate and live birth rate in FET cycles of patients with RIF (Table 2). In addition, we found that intrauterine injection of hCG had a greater effect on improving pregnancy outcomes much more for RIF patients who transferred blastocysts compared with RIF patients transferred cleavage stage embryos (Table 3). The positive effect in this group of patients may be because the uterine exposure time to hCG is lower in patients undergoing blastocyst transfer compared to those having cleavage stage transfer. Furthermore, in this study, the RIF patients who transferred blastocysts were significantly younger than those with cleavage stage embryos. To observe if intrauterine injection of hCG has a greater effect on improving the pregnancy outcomes for younger patients, we conducted a sub-analysis of RIF patients who transferred blastocysts above and below the age of 35. We found that the clinical pregnancy rate, implantation rate and live birth rate were significantly higher in hCG-treated group compared with the control group of patients under 35 years who transferred blastocysts. However, no statistical difference was observed between the study and control groups of patients over 35 years who transferred blastocysts (Table 5). Therefore, intrauterine injection of hCG may be more beneficial in improving the pregnancy outcomes of younger RIF patients.

In addition, the influencing factors of FET pregnancy include age, embryo quality, thickness and type of endometrium, number as well as type of embryo transferred [19]. In this study, age, BMI, EPP, endometrial thickness, number of embryo transfer, number of high quality embryos transferred and embryo type were not significantly different between hCG treated and control groups. In our center, two cleavage stage embryos were cryopreserved and the remaining embryos were cultured into blastocysts if more than five eggs were retrieved. So, the RIF patients who transferred blastocysts usually had good ovarian reserve and were younger than RIF patients transferred cleavage stage embryos. Therefore, the implantation rate was statistically higher in RIF patients transferred blastocysts as compared with RIF patients transferred cleavage stage embryos (Table 4).

We found that hCG could improve pregnancy outcomes of patients with RIF, but the mechanism of this treatment on embryo implantation has not been elucidated. hCG as the earliest molecules secreted by the embryo, is detected as early as 8-cell stage and blastocyst stage before embryo implantation [6, 20]. It can regulate immunological tolerance of the maternal–fetal interface, prolong endometrial receptivity, increase appropriate endometrial angiogenesis, and modulate the endometrial tissue remodelling to promote embryo implantation [3–5].

Tregs have recently been suggested to be necessary for the maintenance of maternal–fetal tolerance [21]. They are widely distributed in peripheral blood circulation and tissues. In humans, there are increasing absolute numbers and percentages of Tregs in normal pregnancy women within circulating lymphocyte populations [22]. Furthermore, elevated Tregs in the peripheral blood is

positively correlated with a better pregnancy outcome in IVF-ET patients [23]. In our previous study, we showed that Tregs reach a peak during the receptive phase at the time of embryo transfer [24]. We assumed that the periodic accumulation of Tregs during the receptive phase may be essential for embryo implantation. Moreover, the percentage of decidual $CD4^+CD25^+CD127^{dim/-}$ Tregs is significantly reduced in patients with recurrent spontaneous miscarriage when compared with controls [25].

hCG increases Treg cells in the periphery and attracts Treg cells to the maternal–fetal interface [4, 26]. Other studies have suggested that hCG has strong immunomodulatory and educates autologous lymphocytes to promote embryo implantation in patients with recurrent IVF-ET failure [27, 28]. Here, we reported that hCG treated patients had a significantly higher percentage of $CD4^+CD25^+CD127^{dim/-}$Tregs in $CD4^+$ T cells than non-hCG-treated patients on the day of embryo transfer, and the pregnancy outcomes was significantly improved in hCG treated group. hCG and LH activate the same receptor, LH/CG-R, which is a transmembrane G protein-coupled receptor [4]. Some data have shown that the LH/CG-R mRNA level was significantly higher in $CD4^+CD25^+CD127^{low/-}$Tregs than in CD4 + T cells and endometrial tissues [16]. We hypothesized that by injecting hCG into the uterine cavity before embryo transfer for RIF patients may improve embryo implantation by increasing the percentage of circulating Tregs.

To exclude the possibility that the local injury resulting from intrauterine administration of hCG induces an inflammatory response that may facilitate the preparation of the endometrium for implantation, the same operation was used in the control group as well as in the study group.

The intrauterine injection of hCG is a simple and safe procedure. This method does not require complex training and is inexpensive. Moreover, the dilution and preparation of hCG does not need special equipment. In this study, the hCG treatment was well tolerated by all patients and produced no side effects.

Conclusions

The intrauterine injection of hCG is a simple and effective method for patients with RIF. We have demonstrated that intrauterine injection of hCG before embryo transfer significantly improved clinical pregnancy rate, implantation rate and live birth rate in FET cycles of patients with RIF by increasing Tregs. For the first time, we have demonstrated that this treatment significantly improves the pregnancy outcomes of blastocyst transfer in young RIF patients.

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Author contribution XML: Project development, Data collection, Data analysis and Manuscript writing. DM: Data collection, Data analysis and Manuscript editing. WJW, QLQ and NZ: the specimen collection and Data management. XRW, JYF, ZM and CFH: Data collection and Manuscript writing.

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Compliance with ethical standards

Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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