



Effects of growth hormone on pregnancy rates of patients with thin endometrium

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Received: 8 December 2017 / Accepted: 19 March 2018 / Published online: 18 April 2018
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Abstract

Purpose To investigate whether growth hormone (GH) could improve pregnancy rates of patients with thin endometrium by clinical study and laboratory experiments.

Materials and methods Ninety-three patients were randomized to either the GH-received group (40) or the routine exogenous administration of estrogens control group (53) for clinical study. The human endometrial carcinoma cell line RL95-2 was used for testing the role of GH with Western blot and real-time PCR by exposure to various concentrations of GH (0.1 nM, 1 nM, 10 nM, 100 nM).

Results Patients treated with GH had a significantly ($P < 0.05$) greater endometrium thickness on day 3 (7.87 ± 0.72 vs 6.34 ± 0.86), higher implantation rates (24.4% vs 10.5%) and greater clinical pregnancy rates (42.5% vs 18.9%) compared with the control group. No adverse events were associated with the use of GH. Administration of GH significantly up-regulated the expression of VEGF, ItgB3 and IGF-I expression in RL95-2 cells at both mRNA and protein levels ($P < 0.05$). AG490, an inhibitor of JAK2, nearly completely inhibited the up-regulative effect of GH through the JAK2-STAT5 pathway, and GH-induced effects could be mediated through autocrine IGF-I together with its hepatic counterpart. IGF-I mRNA was detected in the RL95-2 cells.

Conclusion GH may improve pregnancy outcomes of patients with thin endometrium who undergo frozen embryo transfer by acting on human endometrial cells to promote proliferation and vascularization and to up-regulate receptivity-related molecular expression.

Keywords Endometrial thickness · Growth hormone · Pregnancy rate · Cell experiment · Mechanism

Introduction

In assisted reproductive technology, endometrial receptivity and embryo quality seem to be the two primary factors affecting in vitro fertilization (IVF) and embryo transfer

cycle, and it is reported that nearly two-thirds of failed implantation in IVF are due to reduced endometrial receptivity, with embryo quality to be blamed for the remaining one-third [1]. Endometrial thickness (EMT) is regarded as one of the major influential factors of endometrial receptivity. Some studies showed a positive correlation between EMT on the day of human chorionic gonadotropin (HCG) administration and pregnancy rates, with a significantly ($P < 0.05$) greater EMT in those who had final pregnancy [1]. While there is no consensus on the threshold of EMT sufficient for pregnancy, a cut-off of 7 mm is reported to be related to a compromised pregnancy [2]. The incidence of thin endometrium (< 7 mm) reached approximately 2.4% in IVF patients [3]. Various treatments have been tried to improve EMT including sildenafil [1, 2], L-arginine [4], combination of pentoxifylline and tocopherol [5, 6], and granulocyte colony-stimulating factor [7, 8]. However, there still exist a small number of patients who had thin unresponsive endometrium.

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Growth hormone (GH) could be a potential therapeutic drug for thin endometrium in IVF/ICSI (intracytoplasmic sperm injection). Over the past decades, it has been well known that GH stimulates the expression of insulin-like growth factor-I (IGF-I) via the Janus kinase–signal transducers and activators of transcription (JAK-STAT) signaling pathway, playing a critical role in mediating anabolism on a variety of tissues [9]. GH receptor (GHR) mRNA has been found to localize in human myometrium and leiomyoma [10], suggesting that uterus is also a target tissue. The fact that women diagnosed with acromegaly have higher prevalence of leiomyoma and that women who suffered GH deficiency (GHD) have smaller uterus implied GH directly promoting uterine growth [11, 12]. GH also has a mitogenic effect on endometrial cells, since human GH expression is up-regulated in endometriosis and endometrial adenocarcinoma, and patients with GHR deficiency have lower risk of cancer [13–15]. Furthermore, GHR was found to be expressed in glandular cells of the human endometrium from the mid to late luteal phases throughout pregnancy in the decidua, revealing a key role of GH in implantation [16].

Many studies have reported that GH may support more clinical pregnancies and live births [17–19], however, these studies failed to investigate the effects of GH on endometrium, especially on thin endometrium. We had found an increase EMT in GH-received patients as compared with others in their frozen embryo transfer (FET) cycles in our unpublished study, and we hypothesized that GH could improve pregnancy rates of patients with thin endometrium during FET cycles by enhancing endometrial cell proliferation and improving their endometrial receptivity through the JAK-STAT5 signaling pathway. In this study, we aimed to investigate this hypothesis by means of clinical study combined with in vitro experiments.

Materials and methods

Clinical study

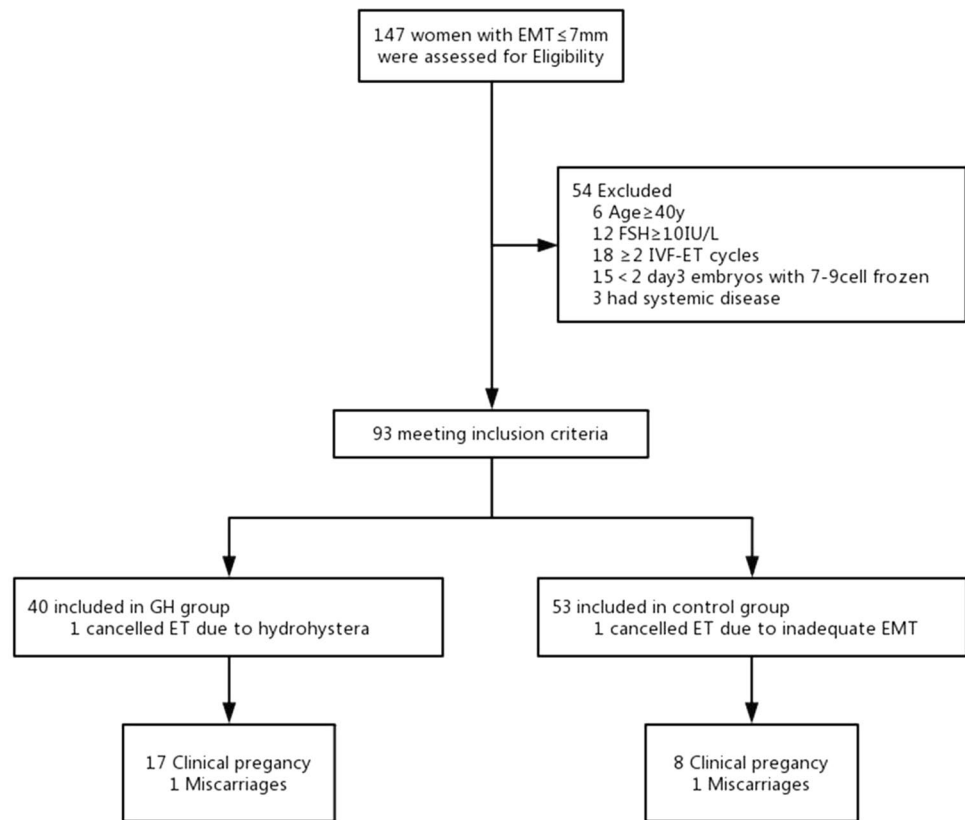
The study was approved by the institutional review board of our hospital, and all the participants had given their written informed consent to participate. Thin endometrium was defined as EMT ≤ 7 mm on the day of HCG administration in their first IVF/ICSI cycle. The inclusion criteria were women aged < 40 years with normal ovarian reserve and thin endometrium (≤ 7 mm) and women who canceled fresh embryo transfer due to EMT ≤ 7 mm on the day of HCG administration in their first IVF/ICSI cycle and who had at least two day 3 embryos with seven–nine cells frozen (Table 1). Women with uterine anomalies, intrauterine adhesions, endometrial polyps, adenomyosis, or a history of malignancy were excluded (Fig. 1). Patients were randomized with a computerized program to either the GH-administered group (40) or the control group with routine exogenous administration of estrogens (53). All patients received oral estradiol valerate (Progynova, Bayer, Leverkusen, Germany) starting on day 3 of their cycles until the 18th day and vaginal estradiol (Femoston, Abbott, Veerweg, the Netherland) 1 mg per day from 15th to 18th day. Estradiol valerate was administered at a dosage of 2 mg twice daily for the first 4 days, and 3 mg twice daily for the following days. Patients in GH group received a daily GH (Saizen, Kinsey, Changchun, China) subcutaneous injection of 5 IU additionally at the same period. The EMT was assessed three times each by two doctors on the 12th, 15th and 18th day of the cycle. If the EMT remained ≤ 7 mm on the 18th day, the patients would be given two choices: cycle cancellation or embryo transfer

Table 1 Demography and treatment results

Variables	GH group (n=40)	Control group (n=53)	P
Age (years)	29.83 \pm 3.01	29.73 \pm 3.58	0.887
BMI (kg/m ²)	22.68 \pm 3.07	23.10 \pm 3.96	0.580
Baseline FSH (IU/L)	7.67 \pm 1.16	7.51 \pm 1.32	0.544
Duration of infertility (years)	3.00 \pm 1.73	2.85 \pm 1.71	0.678
Primary infertility	22/40 (55%)	23/53 (43.4%)	0.268
Times of curettage	0.15 \pm 0.43	0.14 \pm 0.67	0.935
Max. of EMT in history (mm)	5.84 \pm 0.66	5.73 \pm 0.59	0.400
EMT on day 3 (mm)	7.87 \pm 0.72	6.34 \pm 0.86	<0.001
E ₂ level on 18th day (mmol/L)	251.2 \pm 40.3	261.7 \pm 45.2	0.27
High-quality rate of the embryo implanted	35/82 (42.7%)	38/114 (33.3%)	0.182
Miscarriage rate	1/17 (5.9%)	1/10 (10.0%)	0.693
Implantation rate	20/82 (24.4%)	12/114 (10.5%)	<0.001
Clinical pregnancy rate	17/39 (43.6%)	10/52 (19.2%)	0.012

GH growth hormone, BMI body mass index, FSH follicle-stimulating hormone, EMT endometrial thickness, Max maximum

Fig. 1 Flow diagram showing the process of inclusion of patients in the study



despite inadequate EMT. The number of patients whose EMT remained ≤ 7 mm in GH and the control group were 10 and 25, respectively. Only one patient in the control group chose to cancel embryo transfer. Progesterone supplementation was commenced on the 18th day. Two grade II day 3 embryos were transferred on day 4 of progesterone administration. Both groups received intramuscular injection of 60 mg progesterone (Xianju Pharmaceutical, Zhejiang, China) daily combined with 10 mg oral dydrogesterone (Duphaston, Abbott, Veerweg, the Netherlands) twice daily as luteal phase support.

The primary outcome was the EMT on the 18th day. The secondary outcomes were chemical pregnancy, clinical pregnancy and implantation rate. Chemical pregnancy was defined as serum β -HCG > 20 IU/L 14 days after embryo transfer. Clinical pregnancy was defined as the presence of a gestational sac with or without fetal heart examined on ultrasound 14 days after positive β -HCG. Implantation rates were calculated as the number of gestational sacs compared with the number of embryos transferred per group.

Laboratory experiments

Cell line and cell culture

The human endometrial carcinoma cell line RL95-2 was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The RL95-2 cells were cultured according to the recommended conditions.

Cell viability analysis (CCK-8 assay)

Cell viability was analyzed using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Japan). The RL95-2 cells (5×10^3 cells per well) were seeded into 96-well plates in full serum media. Cells were exposed to various concentrations of GH (0.1, 1, 10, and 100 nM) and were cultured at 37 °C in 5% CO₂ for 48 h. CCK-8 was then

added to each well and incubated for an additional 1 h. The absorbance was measured at 450 nm using a microplate reader.

Cell cycle

For cell cycle analyses, the RL95-2 cells were cultured for 48 h in the absence or presence of GH (10 nM) with or without AG490 (50 μ M). Cells were harvested and fixed in ice-cold 70% ethanol overnight at 4 °C. Samples were then centrifuged at 120 g for 5 min. After washed twice with PBS, cells were resuspended in PBS containing 50 μ g/mL propidium iodide (PI) and 0.1% Triton X-100 with 20 μ g/mL RNase A and were incubated at 37 °C for 30 min. All samples were analyzed on a Cytomics FC 500 flow cytometer (Beckman Coulter, Webster, Texas, USA).

Real-time PCR analysis

Total RNA was extracted from the RL95-2 cells with RNAsimple Total RNA Kit (Tiangen, Beijing, China) and then converted to cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan) according to the manufacturer's instructions. The sequences of the primers used in real-time PCR (qPCR) were analyzed in BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and are listed in Table 2. Real-time PCR was performed using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The cDNA was then amplified with SYBR Premix Ex TaqII (Takara, Japan) in triplicate. Triplicate reactions were performed using a two-step amplification program of initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. A melting curve analysis step was added at the end of the amplification, showing no unspecific amplification or primer dimers were existed in the reactions. The $2^{-\Delta\Delta C(T)}$ method was used to analyze the data as described previously. The amount of each gene mRNA was normalized to β -actin transcript level and

expressed as n-fold difference relative to the control. Each change in gene expression was the average of triplicates.

Western blot

The RL95-2 cells were cultured for 48 h in the absence or presence of GH (10 nM) with or without AG490 (50 μ M). The RL95-2 cells were lysed in RIPA lysis buffer with PMSF. 30 μ g of protein lysate was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by transferred to PVDF membranes. The membranes were blocked in fat-free milk for 2 h, and then incubated with the following primary antibodies overnight at 4 °C: mouse monoclonal antibodies anti-VEGF (cat number ab69479; 1:1000; Abcam, Cambridge, MA, USA), mouse monoclonal antibodies anti-IGF-I (cat number I9909; 1:1000; Sigma-Aldrich, St Louis, MO, USA), rabbit polyclonal antibodies anti-ITGB3 (cat number SAB4300526; 1:1000; Sigma-Aldrich, St Louis, MO, USA), rabbit polyclonal antibodies anti-pSTAT5 (cat number SAB4301474; 1:1000; Sigma-Aldrich, St Louis, MO, USA). β -Actin antibody (cat number sc-130300; 1:20 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control. The membrane was then incubated with an HRP-conjugated anti-rabbit or anti-mouse secondary antibody for 1.5 h at room temperature. The signals were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA). The blotting bands were quantified with ImageJ software.

Statistical analysis

Statistical analysis was performed using the SPSS version 21.0 software package (SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean \pm standard deviation (SD). Differences between two groups were checked by Student's *t* test (continuous variables) or by χ^2 test and Fisher's exact test where needed (categorical variables). Differences between multiple groups were evaluated by one-way

Table 2 Primer sequences (all 5'–3') used in real-time RT-PCR

Gene	Gene bank accession no.	Forward primer (reverse primer)	Amplicon size (bp)
Homo ITGB3	NM_000212	GTGACCTGAAGGAGAATCTGC (CCGGAGTGCAATCCTCTGG)	161
Homo LIF	NM_002309	CCAACGTGACGGACTTCCC (TACACGACTATGCGGTACAGC)	82
Homo ITGAV	NM_001145000	ATCTGTGAGGTGCGAAACAGGA (TGGAGCATACTCAACAGTCTTTG)	136
Homo HOXA10	NM_018951	TGGCTCACGGCAAAGAGTG (GCTGCGGCTAATCTCTAGGC)	129
Homo VEGFA	NM_001171627	AGGGCAGAATCATCACGAAGT (AGGGTCTCGATTGGATGGCA)	75
Homo EGF	NM_001178131	TGGATGTGCTTGATAAGCGG (ACCATGTCCTTTCCAGTGTGT)	223
Homo IGF1	NM_001111283	GCTCTTCAGTTCGTGTGTGGA (GCCTCCTTAGATCACAGCTCC)	133
Homo SPP1	NM_001251830	CTCCATTGACTCGAACGACTC (CAGGTCTGCGAACTTCTTAGAT)	230
Homo β -actin	NM_001101	CATGTACGTTGCTATCCAGGC (CTCCTTAATGTACGCACGAT)	250

ANOVA with a post hoc test of Tukey–Kramer. A P value <0.05 was considered statistically significant.

Results

GH improves endometrium thickness, implantation rates and clinical pregnancy rates of patients with thin endometrium

Baseline characteristics of the patients are showed in Table 1. No difference was observed between the two groups in age, body mass index, basal FSH level, infertility duration, proportion of primary infertility, times of curettage, the maximum of endometrium thickness in history and the quality of embryo implanted. Patients treated with GH had a significantly ($P < 0.05$) greater endometrium thickness on day 3, higher implantation rates and greater clinical pregnancy rates compared with the control group (Fig. 2). No adverse events were associated with the use of GH.

GH promotes RL95-2 cells proliferation and activates cell cycle in vitro

The RL95-2 cells were incubated with various doses of GH in CCK-8 assay. A dose-dependent increase of viability in the RL95-2 cells was observed, with the maximum viability at 10 nM GH (Fig. 3). Compared to the control group (A), GH induced a dramatic increase in percentage of RL95-2 cells in S phase [(28.62 ± 1.45) vs. $(38.32 \pm 0.98)\%$, $P < 0.05$] and a marked decrease in G0/G1 phase [(60.98 ± 1.71) vs. $(48.26 \pm 0.73)\%$, $P < 0.05$] (Fig. 4). No significant ($P > 0.05$) difference in cell proportion was detected between group A, C or D).

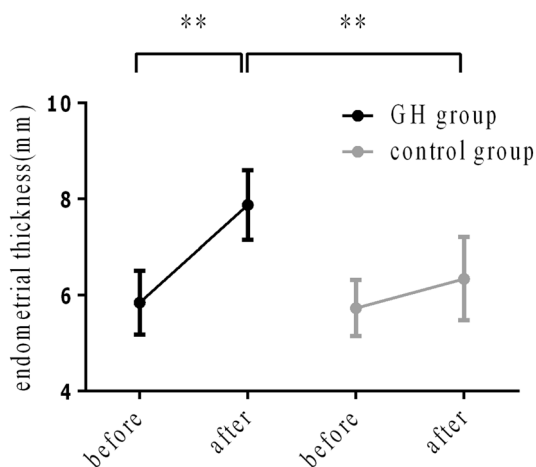


Fig. 2 Alternation of endometrial thickness in the growth hormone (GH) group and control group (** $P < 0.01$)

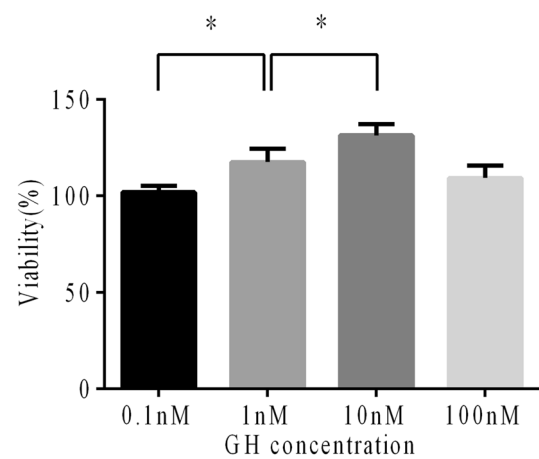


Fig. 3 Cell proliferation assay (CCK-8 assay). The cells were exposed to growth hormone (GH) of 0.1, 1, 10, 100 nM for 48 h (* $P < 0.05$)

GH up-regulates the expression of VEGF, ItgB3 and IGF-I in RL95-2 cells

Our data found that GH significantly up-regulated VEGF, ItgB3 and IGF-I expression in the RL95-2 cells at both mRNA and protein levels ($P < 0.05$) (Figs. 5, 6). AG490, an inhibitor of JAK2, nearly completely inhibited the up-regulative effect of GH, suggesting that the JAK2-STAT5 pathway was involved in this upregulation process (Fig. 6). No impact of GH on the expression of ItgAV, LIF, EGF, HOXA10 and SPP1 was demonstrated in the experiment. IGF-I mRNA was detected in the RL95-2 cells, indicating that GH-induced effects could be mediated through auto-crine IGF-I together with its hepatic counterpart.

Discussion

In this study, we investigated whether GH had proliferative and implantation-facilitating effects on thin endometrium. We performed a clinical study in combination with in vitro experiment to clarify the effect and possible mechanism. Our results indicated that GH supplement can improve pregnancy outcomes of patients with thin endometrium in FET. Furthermore, the mechanism may involve GH which can act in a direct or IGF-I mediated manner in human endometrial cells to promote proliferation and vascularization and to up-regulate receptivity-related molecular expression.

Casper speculated that poor EMT has a direct detrimental effect on implantation due to higher oxygen tension of the surface endometrium [20]. Our findings indicated that GH can promote endometrial cell proliferation in vitro and increase the EMT in patients with thin endometrium. This might be one of the reasons why the GH-received group has a better outcome. A case report revealed that GH supplement

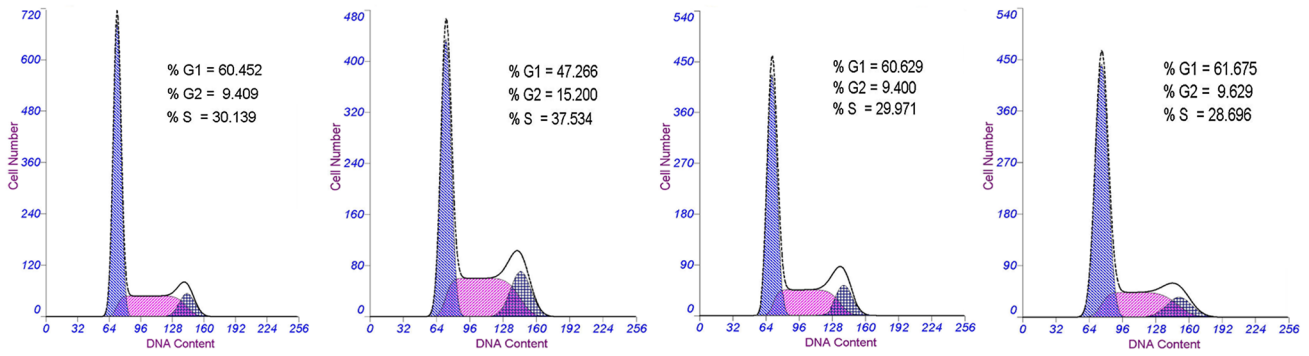


Fig. 4 Flow cytometric analysis of the cell cycle. The RL95-2 cells alone (a), the RL95-2 cells+GH 10 nM (b), the RL95-2 cells +GH 10 nM+ AG490 50 μM (c), AG490 50 μM (d). Cell cycle analyses were performed after 48 h

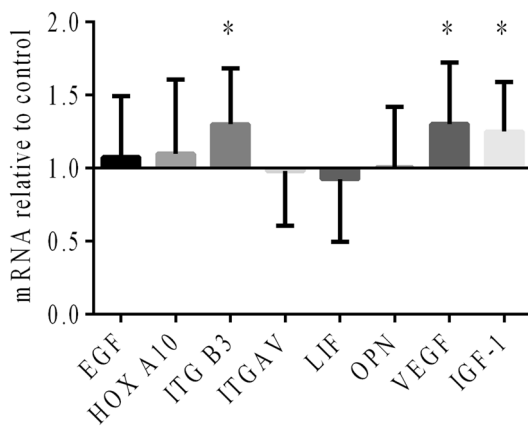


Fig. 5 Analysis of eight receptivity-related genes in RL95-2 cells (**P* < 0.05) in real-time PCR

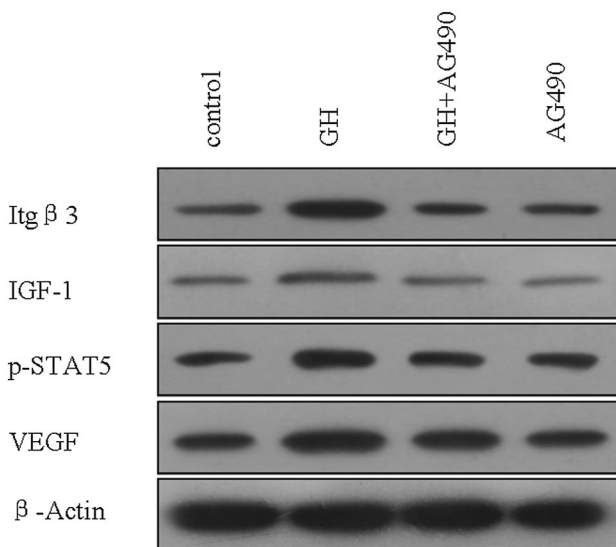


Fig. 6 Effects of GH and/or AG490 on Itgβ3, VEGF and JAK/STAT pathway-related protein expression

in a panhypopituitarism woman resulted in an acceptable endometrium and live birth [21]. However, Rajesh et al. [22] indicated that GH supplement to GHD patients in IVF cycle did not seem to increase their EMT. This discrepancy may be partly due to the GHR disorder of GHD patients, since insufficient nuclear GHR is also related to lower proliferative status [23].

Promoted vascularization also facilitated pregnancy. The state of high blood flow resistance and down-regulated VEGF expression with inadequate epithelial growth and vascularization were regarded as the pathophysiologic characteristics of thin endometrium [24]. It has been demonstrated that subendometrial blood flow on the day of embryo transfer is relevant to the implantation and pregnancy rate in IVF cycle [25]. It is widely acknowledged that VEGF plays an important part in angiogenesis [26]. A recent study reported that VEGF can act in an autocrine manner on endometrial epithelial cell adhesion as a key regulator in the implantation progress [27]. Our results showed that VEGF expression was up-regulated in endometrial cells when exposed to GH, consistent with that reported by Brunetdunand et al. [28], who found that autocrine hGH may increase VEGFA expression in cancer cells. A similar observation has been documented that endogenous GH may have a positive effect on uterine receptivity at the time of implantation [29]. Up-regulated VEGF in the GH group partly resulted in the increase of subendometrial blood flow, thus improving pregnancy outcomes.

Additionally, integrin β3, a generally accepted biomarker of uterine receptivity [30], was increased in the GH group. It has been demonstrated that decreased endometrial αγβ3, which can be found in patients with unexplained infertility, endometriosis and luteal deficiency, is related to lower pregnancy rates [31, 32]. This provides evidence that GH may have a positive effect on the improvement of endometrial receptivity and pregnancy outcomes. Recently, a similar observation has been documented that GH may improve

uterine receptivity of RIF patients, further confirming our results [33].

High estrogen levels do have blocking effects on IGF-1 generation [34, 35]. However, in our study, estrogen was equally administered in both groups, and the estrogen levels were not significantly ($P > 0.05$) different in both GH (251.2 ± 40.3 mmol/L) and control (261.7 ± 45.2 mmol/L) groups (Table 1), resulting in similar blocking effects on IGF-1. The difference of IGF-1 levels between the two groups was largely due to supplementation of GH. Previous studies have reported that IGF-1 concentrations in serum and follicular fluid were significantly elevated in the GH-treated women [36, 37]. It was noted that IGF-I expression on the RL95-2 cells was enhanced after GH exposure in vitro, indicating that autocrine IGF-I also plays a vital role [38]. Our results confirmed the findings by Hull et al. [39] that GH-induced autocrine IGF-I may be more important than its hepatic counterpart in the corresponding GH-target tissues. IGF-I may contribute to endothelial progenitor cells differentiation and induce vascular genesis [40]. Furthermore, a variety of study demonstrated that IGF-I is a mediator in the progress of E2 acting on uterus and that IGF-I may have an impact on ER function [41]. Others have shown that IGF-I, IGF-II and EGF mRNA are expressed in endometrium [42] and these factors may play a critical role in steroidogenesis [43]. Up-regulated IGF-I after GH supplement may promote the effect of E2 on endometrial cells, thus contributing to the increase of EMT.

JAK2/STAT5 has been known to be the major pathway to mediate the metabolism-regulated function of GH in bone, liver and adipose tissue [9]. It is reasonable to hypothesize that the JAK2/STAT5 pathway is also indispensable for GH in endometrium, but no studies have been conducted to confirm this so far. However, our study supported this hypothesis.

Although autocrine GH was found up-regulated in endometrioid adenocarcinoma [15], the proliferative effect of GH is not equivalent to oncogenic effect. Exogenous and endocrine GH does not appear to increase cancer risk, for the fact that the cancer incidence is normal in patients suffering from acromegaly and GHD who need GH supplement [44, 45]. This discrepancy may be partly caused by differences in gene expression and cell growth of autocrine GH [46]. Other studies revealed that chronic GH over-exposure may increase the incidence and severity of diabetes mellitus, which has been found in patients with acromegaly [47, 48]. However, GH is administered at a lower dose with a shorter duration in treating thin endometrium and there have been no adverse effects reported up to date.

While lots of studies about GH in the assisted reproductive technology focused on ovary [49, 50], there are relatively fewer studies exploring possible effects of GH on endometrium. To the best of our knowledge, this is the first

study to explore the underlying mechanism by cell culture. Our results offered the first evidence that GH can act in a direct or IGF-I mediated manner in human endometrial cell to promote proliferation and vascularization and to up-regulate receptivity-related molecules expression and that the JAK/STAT pathway may be involved.

Although our data are encouraging, the number of patients in our study was limited owing to the low incidence of thin endometrium. So, the effects of GH on patients with thin endometrium need to be further confirmed. Another limitation of our study is that we used the RL95-2 cell line for endometrial specimens to avoid collecting specimens in luteal phase.

In conclusion, GH supplement may improve pregnancy outcomes in patients with thin endometrium who underwent FET through the action of GH on human endometrial cells to promote proliferation and vascularization and to up-regulate receptivity-related molecular expression via the JAK/STAT pathway. Understanding the underlying mechanism of GH would be of great value for the treatment of thin endometrium, making GH supplement in FET cycle a promising management. Further studies are needed to elucidate how GH modulates endometrial epithelial cells to the attachment and expansion of trophoblast spheroids.

Funding This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Compliance with ethical standards

Conflict of interest We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee 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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