GROWTH HORMONE IN FERTILITY AND INFERTILITY: PHYSIOLOGY, PATHOLOGY, DIAGNOSIS AND TREATMENT

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GROWTH HORMONE IN FERTILITY AND INFERTILITY: PHYSIOLOGY, PATHOLOGY, DIAGNOSIS AND TREATMENT

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EDITORIAL
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Editorial: Growth Hormone in Fertility and Infertility: Physiology, Pathology, Diagnosis and Treatment

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Keywords: growth hormone, fertility, infertility, physiology, pathology, diagnosis, treatment

Editorial on the Research Topic

Growth Hormone in Fertility and Infertility: Physiology, Pathology, Diagnosis and Treatment

INTRODUCTION

Growth hormone (GH) has been used in the treatment of infertility since the late 1980s (reviewed in Homburg and Ostergaard (1)). However, its indication was mostly empirical, rather than scientifically founded, but with clearest benefits in women with hypopituitarism One of the first prospective randomized studies showed a positive effect of GH administration on delivery and live birth rates in women aged >40 years treated by intracytoplasmic sperm injection (ICSI) (2). Since then GH was used mainly in older women. However, more recent studies have suggested the possibility of a beneficial effect of GH on *in vitro* fertilization (IVF) outcomes even in some younger women with previous unexplained IVF failures (3). Moreover, the administration of GH to women receiving embryos from donated oocytes improved results in some cases, showing that GH can also have a positive effect on uterine receptivity (4).

GH is mainly secreted by the pituitary gland, but local production in various organs, including the ovary, has also been demonstrated (5). In addition to acting through its own receptors, some effects of GH can also be mediated by GH-induced insulin-like growth factor-I (IGF-I) gene activation (6).

The aim of this Research Topic was to analyze the effects of GH on the female reproductive function in different clinical scenarios, with a special accent on distinguishing those women who would benefit from GH administration from those who would not. Even though all biological and clinical aspects of GH action in the female reproductive system still remain unknown, the data presented in this series of papers have brought us closer to rational strategies of GH indication replacing purely empirical and blind indications.

THE MAIN POINTS OF INDIVIDUAL CONTRIBUTIONS

This series includes 13 papers: five original research articles and eight review articles, three of which focus on the complexity of GH physiology. In this section, they are referred to in a chronological order as they were published in the Journal. In their review article, Dosouto et al. present data from animal models and clinical trials showing that both GH and IGF-I are synthesized locally in the ovary, and the synthesis of IGF-I can be stimulated locally by other factors than GH, such as steroid

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hormones and gonadotropins. The original research paper by Li et al. shows that the addition of GH (200 ng/ml) to the culture medium improves oocyte maturation from the germinal vesicle stage to the metaphase II stage as well as their postfertilization developmental competence. The review article by Devesa and Caicedo highlights the potential role of ovarian angiogenesis as a mediator of GH effects on follicular development and oocyte quality. Xu et al. review scientific reports on the effect of GH on IVF outcomes, focusing on differences between those reporting a positive effect (mostly related to oocyte and embryo quality) and those in which no clinical benefit was demonstrated. The original research article by Shi et al. compares a retrospective single-center cohort analysis of 18,455 IVF cycles, including the transfer of fresh and frozen embryos, performed in good and low prognosis patients, and analyze how the cumulative live birth rate is influenced by the patient's age, antral follicle count, and the number of oocytes obtained, respectively. This is one of three articles applying the Poseidon algorithm in defining poor-prognosis patients with poor ovarian reserve. Cai et al. present an original research article analyzing, retrospectively, the effect of GH in patients with poor ovarian reserve defined by the Poseidon algorithm. They show that GH pretreatment elevates ovarian response to stimulation, improves live birth rate, and reduces miscarriage rate in this group of patients. The review article by Yovich et al. compares their own experience at the PIVET Center (Australia) with that of the total of 42 GH studies performed since the year 2000 all over the world. They conclude that GH increases both oocyte and embryo utilization rates in most cases, but only ~50% are followed by elevated live birth rates. Lee et al., in their original research article, demonstrate that even low dose GH adjuvant treatment improves pregnancy outcomes in poor responders provided that it is combined with an ultra-long ovarian stimulation protocol, particularly in women under 40 years of age. In their review article, Yovich et al. resume the modes of action of GH and IGF-I in the ovary and suggest GH/IGF deficiency as the main cause of decreasing fertility in older women. They also add some original data suggesting how women needing GH support can be identified. The review article by Ipsa et al. presents a comprehensive overview of the molecular mechanisms of GH and IGF action in reproductive tissues, pointing out different interesting topics for future research. Two mini-reviews authored, respectively, by Altmäe and Aghajanova and Liu et al. deal with the effect of GH on endometrial receptivity. The former analyzes the potential molecular mechanisms of GH action in the endometrium, while the latter focuses on the current clinical experience. The last article of this series is an original research article by Tesarik et al. showing that GH administration also improves oocyte, zygote, and embryo quality, as well as the clinical IVF outcomes, in young women with previous repeated implantation failures. Although not included in this Research Topic, we would add the contemporary publication of Regan et al (7). which reported clear beneficial effects of GH on various receptors for reproductive hormones, as well as improving the profile of expression of these receptors in association with successful clinical outcomes in older women.

SYNTHETIC VIEW AND CONCLUSIONS

Taken together, the data presented in this Research Topic touch almost all aspects of GH effects on female fertility, both the biological ones and the clinical ones. Some of these data help identify women who are likely to benefit from GH treatment and distinguish them from those who are not. Other data show the ways for future biological and clinical research to further improve the scientific basis of GH indication.

AUTHOR CONTRIBUTIONS

All three authors (editors) contributed equally to the manuscript preparation. All authors contributed to the article and approved the submitted version.

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REVIEW
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Growth Hormone and Reproduction: Lessons Learned From Animal Models and Clinical Trials

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Growth Hormone (GH) has been considered as a therapeutic option to increase the number of growing follicles during Assisted Reproductive Technology (ART) for more than 30 years. In this review the biological rationale for therapeutic GH usage is explained through evidence in animal models, aiming to put this into a clinical context. First, we explain the GH—Insulin like Growth Factor (IGF)-1—gonadal axis and its role in reproduction. Evidence suggests that GH can stimulate the secretion of IGF1 not only in the liver but also in the peripheral target structures, including the ovary. Moreover, IGF-1 can be secreted locally under the influence of stimuli other than GH. In the case of the ovary, steroid hormones, gonadotropins or the combination of both seems to be involved. Even more interesting, the ovary itself can secret GH locally and exert a paracrine action modulating the intracellular signaling pathway of GH, i.e., not by the systemic pathway where GH binds to the extracellular domain of the GH receptor. Finally, these aspects from animal models are put into clinical perspective by discussing results and shortcomings of studies and meta-analyses in order to put forth the state-of-the-art rationale for therapeutic GH usage in modern ART.

Keywords: growth hormone, infertility, poor ovarian response, POSEIDON, IVF

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INTRODUCTION

GH is a monomeric protein secreted by the pituitary with a high molecular similarity to other lactogenic hormones like prolactin and placental lactogen. In the anterior pituitary gland, the secretion by the somatotroph cell is regulated by both stimulatory peptides [e.g., Growth Hormone Releasing Hormone (GHRH)] and inhibitory (e.g., Somatostatin) peptides. The secretion takes place in a pulsatile way that combines short-term variability of spikes of irregular amplitudes with a clear circadian increase, coinciding with the late non-Rapid Eye Movement (REM) periods, probably mediated by dopamine related neurotransmitters (1, 2). This complicates the determination of optimal plasma levels. The action of GH is exerted through its binding to the extracellular domain of a complex membrane receptor. In contrast to dimeric glycoproteins like gonadotropins, two receptors are needed in order to establish a trimeric structure composed by two membrane receptors and the GH molecule. Thus, three recognition processes are needed for an effective downstream activation: receptor-to-receptor and agonistic GH molecule to each of the receptors to form the activated GH trimeric complex. This complex relationship between the hormone and the target organ makes the process of activation vulnerable to different mutations, causing different downstream effects such as the clinical diversity in the different phenotypes of e.g.,

dwarfism (3, 4). The classic paradigm establishes that pituitary GH acts on its hepatic receptors and stimulates the secretion of somatomedins or Insulin-like growth factors (IGFs): insulin like growth factor 1 and 2 (IGF-1 and IGF-2). These are molecules sharing near 50% homology with pro-insulin. However, IGF-1 and IGF-2 seem to have different roles. While IGF-1 is considered the mediator of the classical biological actions on growth, development and cellular proliferation, IGF-2 is relevant in the regulation of perinatal development. The secretion of IGFs is induced by GH mediated activation of single copy genes. IGF mRNAs have been detected in several target tissues, and at the same time both IGF types exert a negative feedback at the hypothalamic level maintaining basal steady levels of GH (1). Both circulating and local bioavailability is regulated by high affinity binding proteins which fine-tune their local action (5).

NEW EVIDENCE FROM ANIMAL MODELS

In the last decade the old paradigm described above has been challenged by new evidence obtained in genetically manipulated research animals, introducing new elements of complexity to be taken into account when interpreting the role of GH in any peripheral structure, especially in the ovary. As reviewed thoroughly by others (6), GH can stimulate the secretion of IGF-1 not only in the liver but also in peripheral target structures (6). Moreover, IGF-1 can be secreted locally under the influence of stimuli other than GH. In the case of the ovary, steroid hormones, gonadotropins or the combination of both can be involved. Finally, the ovary itself can secret GH locally and exert a paracrine action, modulating the intracellular signaling pathway of GH and this occurs without binding to the extracellular domain of the membrane GH receptor. This is especially relevant since, contrary to the pituitary secretion, the ovarian secretion of GH takes places in a regular, non-pulsatile, non-circadian pattern.

In GHR knockout mice, circulating GH levels are high and IGF-1 levels are low (7). All studies on blocking or impairing the action of GH on its receptor report a delay in puberty, a significant reduction in litter size (a mean from 6, 7 in wild type animals to 2, 7 in transgenic) (8), and a corresponding delay in the exhaustion of the follicular pool (3). However, the genetically modified animals are fertile and deliver small litters of healthy animals. The experimental data show that this decrease in litter size is the consequence of a reduction in ovulation rate rather than problems related to implantation failure or early embryo loss. Histological examination of the ovaries shows an increase in primordial or primary follicles and a decrease in the number of healthy and growing antral or pre-ovulatory follicles (7–10). It is difficult to establish to which extent this is the result of abnormal GH signaling or its immediate downstream mediator, i.e., a decrease in local IGF-1 secretion. Interestingly, the negative effects seen in the abovementioned studies can be reverted by the administration of IGF-1 (11).

A suitable model to clarify the specific role of IGF-1 is the IGF-gene knockout mouse (12). In these mice, the lack of expression of IGFs results in dwarfism and infertility. The female mutated animals fail to ovulate either spontaneously or under the influence of gonadotropins, proving the importance of IGF1 in the progression of cohorts from primordial and primary stage to recruitable secondary follicles and in the sensitivity to gonadotropins during the process of selection and follicular growth. Interestingly, the histological observation shows an increase in primordial and primary follicles as compared to the wild type animal and an absence of antral follicles. These findings reinforce the idea of a crucial role for IGF in the process of progression of the follicles from the non-gonadotropin sensitive to the gonadotropin sensitive stages.

Shiomi-Sugaya et al. observed that in an "*in vitro*" model of secondary follicles from mice isolated in gel media, the growth rate of the follicles or their time to atresia correlate with IGF-1 mRNA expression (13). Also interesting is the relationship between IGF-1 mRNA and the presence of theca cells. Follicular progression was arrested by blocking IGF-1 production and restored by the co-culture with the cytokine, thus confirming the importance of IGF-1 in follicular development. These observations suggest a peri-follicular microenvironment where theca cells, beyond providing precursors for local estrogen production, modulate follicular progression through paracrine action of androgens and IGFs.

A completely different approach to study GH role on follicular dynamics is based in modifying GH secretion at the pituitary level (14). In GH df/df Ames dwarf mice GH pituitary secretion is practically abolished. In this context the pool of primordial follicles is clearly increased as compared to N/df or wild type animals. GH administration reverses this situation and decreases primordial follicular count while increasing the number of antral follicles. On the contrary, transgenic mice overexpressing GH have a reduced number of primordial follicles as compared to controls. It is relevant to note that, in the wild type animals, the administration of GH diminishes the primordial follicular population but do not increase the number of antral structures, probably due to a subsequent increased atresia rate. Taken together, these findings suggest that in the absence of GH, follicles remain in the primordial stage.

EVIDENCE FROM "HUMAN MODELS"

In humans the evaluation of GH role on reproduction can be approached through two different models: GHRH or GH receptor mutations or combined pituitary hormone deficiencies (CPHD). In the Itabaianinha County, in Brazil, there is an ethnic group with high prevalence of a mutation of the gene encoding GHRHR gene, resulting in a severe reduction in GH signaling. Beyond the phenotypic characteristics of GH deficiency, the affected individuals have delayed puberty, but are fertile mimicking what is found in animal models with GH deficiency (15).

Similar clinical findings are observed in a group of predominantly Sephardi Jewish with up to 29 mutations of the gene encoding for GHR (16). A cohort of seven married women has been followed for their entire reproductive lives. Five of them have conceived 11 term pregnancies

and 4 miscarriages. All pregnancies were spontaneous except for one that was obtained by IVF. Six women reached menopause between 48 and 51 years. In a different model of childhood onset CHPD, Correa reports on five cases from a single center. Pregnancy has been obtained in all cases with controlled ovarian stimulation and GH –LT4 co-treatment (17).

SUMMARY: LESSONS LEARNED FROM ANIMAL AND HUMAN MODELS

All experimental or clinical situations of early GH/IGF deprivation result in a delay in pubertal development with a corresponding prolongation of reproductive life. Microscopically there is a change in the composition of the follicular pools with a predominant population of primordial and primary follicles and the absence or limited presence of more advanced stages of follicles. This situation can be reverted with GH or IGF-1 administration. IGF-1 has proven to be necessary for ovulation to occur while in the absence of GH follicular development, ovulation, and pregnancy can take place. In these cases, however, the size of the litter is significantly decreased. All these findings suggest a significant role of both systemic and local GH/IGF-1 regulation in the progress of follicles from non-gonadotropin to gonadotropin dependent status and also in improving follicular development and oocyte maturation. Thus, it is biologically plausible that GH administration can play a positive role in increasing the number of recruited follicles, especially in cases with limited ovarian reserve. If the evidence from animal models can be directly translated into humans, the administration of GH with the purpose of improving the oocyte yield should begin earlier than the stimulation with gonadotropins. In the same line of thinking Gleicher and colleagues explore this hypothesis, www.*clinicaltrial.gov (NCT02179255),* suggesting to initiate HGH at least 6 weeks prior to start of COS.

Figure 1 shows factors influencing the dynamics of follicular development. Activators such as GH and IFG-BP (*Insulin Growth Factor Binding Protein)* complexes, Insulin, androgens and activin, might promote follicular growth, transition to antral stage or even follicular recruitment, either by acting as anti-apoptotic factors or enhancing follicular response to gonadotropins. Inhibitors such as *Anti Müllerian Hormone* (AMH) are able to block initial follicle recruitment, transition to antral stage or even the gonadotropin-dependent recruitment. Late follicular stages are predominantly influenced by endocrine factors such as gonadotropins: mainly FSH in the recruitment and selection of the leading follicle and LH at later stages and last oocyte maturation and ovulation. Although there has been a clear differentiation between gonadotropin-independent and gonadotropin responsive/dependent stages, all the molecules mentioned have been shown to take part not only at one level, but in the entire process of folliculogenesis. Basic science studies provide biologically plausible data for GH and IGF-1 as key factors for an optimal follicle development. GH may play an activating role, either directly or indirectly, via for instance IGF-1 in the transition from primordial follicles to late antral stages.

FIRST CLINICAL EVIDENCE OF THERAPEUTIC GH IN IVF

In the late eighties, a few case studies in patients undergoing COS for IVF and ovulation induction (OI) suggested that administration of GH improved the ovarian response to stimulation with gonadotropins (19–21). Later it was shown that GH treatment was associated with minor adverse reactions, mainly gastrointestinal symptoms, in ∼17% of cases (8/48) (22). The first reported mechanism by which GH would enhance FSH action was by up-regulating the synthesis of IGF-1 in granulosa cells (23). Animal studies suggested that GH increased the intra-ovarian synthesis of the IGF-1 *in vivo* and *in-vitro* (24, 25) and that this interaction was an important part of ovarian physiology in humans (26, 27). Addition of IGF-1 in granulosa cell cultures increased the intrinsic action of gonadotropins by enhancing aromatase activity, estradiol (E2) and progesterone (P) production and LH receptor formation (27, 28) and was able to stimulate follicular development and oocyte maturation (25).

In 1990, a well-designed controlled clinical trial confirmed the synergistic effects of GH in patients undergoing IVF and stimulated with human menotropin gonadotropin (HMG) (21). However, another RCT with 20 suboptimal responder patients concluded that there was no improvement in the ovarian response by adding GH although there was a trend for more developing follicles ($P = 0.06$) (29). Interestingly, a sub-analysis of this study in patients with polycystic ovaries (PCO) showed a significant increase in the number of follicles developed ($P = 0.04$) and the number of oocytes retrieved ($P = 0.03$). The study did not report pregnancy rates and live birth rates (LBR) (29). Another study focusing on polycystic ovarian syndrome (PCOS) patients as a target for GH treatment found favorable responses in terms of serum and follicular IGF-1 concentrations (30). Despite not reporting conclusive clinical results, these and other early studies reported that GH treatment seemed to promote ovarian steroidogenesis and follicular development (22).

META-ANALYSES: A NEED FOR FURTHER RESEARCH

In 2003, a Cochrane review and meta-analysis concluded that the use of GH in COS for IVF was in need of further research (31). The meta-analysis covered studies with GH co-treatment administered in varying dosages (4, 8, and 12 mg) and with intervention performed alongside stimulation start. There were no significant differences in any outcome measure and at any of the dosages used. Following this meta-analysis, five subsequent meta-analyses assessed the clinical use of GH as adjuvant in IVF. The first analysis reported an increase in the clinical pregnancy rates (CPR) and LBR by the administration of GH during COS with gonadotropins in PORs—an absolute increase in CPR by 16% (95% CI: +4 to +28; fixed effects model) (number-needed-to-treat = 6, 95% CI:4–25). Moreover, GH supplementation was associated with a significantly higher proportion of patients reaching embryo transfer (32). Despite this promising result, the total number of cases included in the meta-analysis was too small

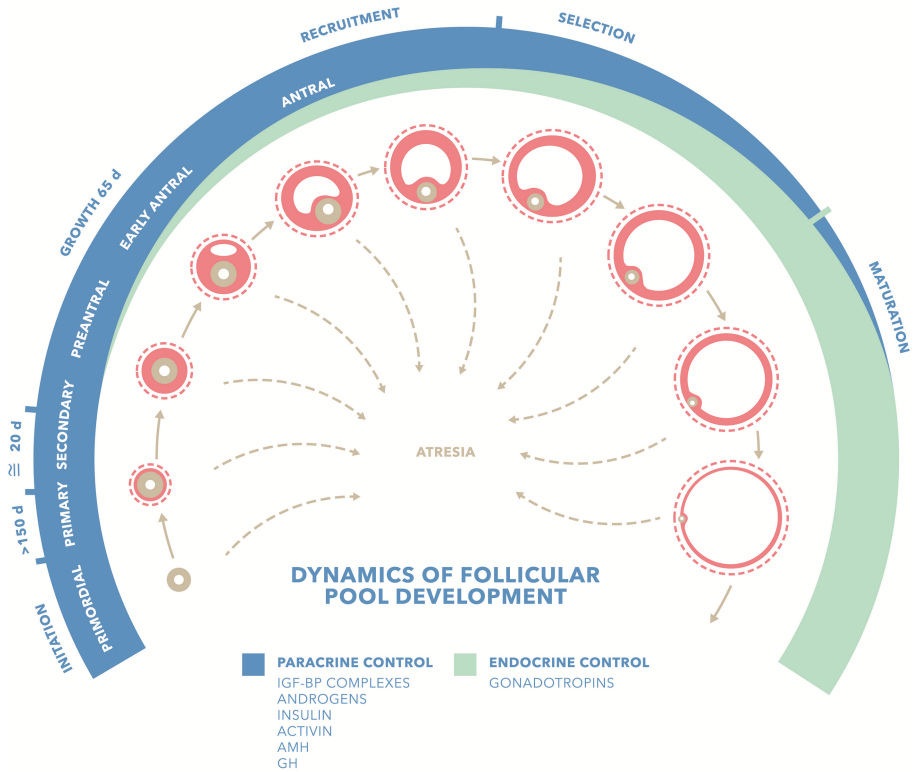

FIGURE 1 | Factors influencing dynamics of follicular pool development. Adapted from Gougeon (18) *IGF-BP complexes (Insulin Growth Factor Binding Protein Complexes); GH (Growth Hormone); AMH (Anti Müllerian Hormone)*. Blue stripe shows follicular stages predominantly influenced by paracrine factors: activators such as GH and IFG-BP complexes, insulin, androgens and activin and AMH. Green stripe shows follicular stages predominantly influenced by endocrine factors such as gonadotropins. Although there has been so far a clear differentiation between gonadotropin-independent and gonadotropin responsive/dependent stages, all these molecules have shown to take part not only at one level, but in the whole folliculo-genesis.

to reach robust evidence (only 169 patients in a total of 6 RCTs). It is important to stress that from the 2003 Cochrane review to the meta-analysis by Kolibianakis by (32), only one well designed RCT was published in the literature, comparing the use of GH alone as an adjuvant to COS in PORs (33). The study involved 61 PORs patients, and the study group ($n = 31$) received daily GH co-treatment (4 mg subcutaneously) mg from the first day of GnRHa down regulation (day 21 of the preceding cycle) until the day of the ovulation trigger (OT). The control group ($n = 30$) received the same protocol except for the GH co-administration. A numerically higher CPR was achieved in the GH group (12/31) as compared to the control group (6/30). However, this difference did not reach statistical significance. Prior to that two Chinese studies were conducted in PORs and investigating the use of GH (34, 35). Both studies were only available in full-text in Chinese, hence, we did not include them in this review.

Kyrou et al. (36) performed a meta-analysis of RCT's which evaluated interventions aiming at increasing pregnancy rates in PORs. The only adjuvant treatment to standard stimulation that appeared to increase the probability of live birth was the addition of GH (OR 5.22, CI: 95% 1.09–24.99). Later, Duffy et al. (37) made a revised update of the Cochrane meta-analysis, including 10 studies with a total of 440 patients. Results demonstrated a statistically significant difference in LBR favoring co-administration of GH in IVF protocols in POR patients without increasing adverse events (OR 5.39, 95% CI 1.89–15.35). Notably, most of the studies included in these meta-analyses led to a potential bias in the results due to poor description of the method of randomization. Moreover, there were significant differences in timing and dose of GH co-administration as well as high heterogeneity in the definition of POR.

In 2015, Yu et al. (38) performed an updated meta-analysis reporting results in line with previous analyses. The results showed a significant improvement in terms of metaphase II oocytes retrieved, number of 2PN obtained and number of embryos available for transfer by GH supplementation in IVF patients. However, no difference was seen as regards CPR.

THE SEARCH FOR THE MOST OPTIMAL STIMULATION PROTOCOL

A retrospective matched case–control study including 42 patients explored for the first time the effect of GH as an adjuvant in a micro-dose GnRH agonist flare-up protocol. The study group was treated with 3.33 mg GH daily subcutaneously (SC) for 14 days before starting COS (39). The authors did not find differences in any of the reported outcomes, although the

small sample size and the retrospective character of the study necessitated a future RCT to draw firm conclusions. A RCT including a total of 141 patients was subsequently performed in GnRH antagonist co-treated Bologna criteria POR patients (40). In this study, GH administration was initiated on day 6 of hMG stimulation in a daily dose of 2.5 mg SC until the day of HCG trigger. The study group had significantly fewer days of stimulation, more oocytes retrieved and better fertilization rates, albeit the authors did not find significant differences in CPR per cycle and LBR per cycle. These results were similar to those previously published in the first RCT using GH during GnRH antagonist co-treatment (41). In 2015, an open label four arm randomized study including a total of 287 POR patients aligned with the ESHRE Bologna criteria aimed at comparing 4 different stimulation protocols (42). All groups were administered GH on day 6 of hMG stimulation in a daily dose of 2.5 mg SC. Patients were randomly allocated to either a long or short GnRH agonist protocol, mini-flare or GnRH antagonist protocol. The long protocol was superior regarding the number of oocytes retrieved and fertilization rate, although no differences were seen in CPR. More recently, Dakhly et al. explored GH adjuvant treatment in the long agonist protocol in a prospective randomized study with 240 patients (43). The intervention group received adjuvant GH co-treatment 2.5 mg s.c (7.5 IU) from day 21 of the previous cycle along with GnRHa, until the day of HCG trigger. Authors found statistically significant differences in terms of number of oocytes collected in favor of GH [(5.4 $\pm$ 1.7) vs. 4.3 $\pm$ 2.1], but they failed to show statistical differences in LBR in both fresh (17.5 vs. 14.1%) and cumulative frozen embryo transfer cycles (18.3 vs. 14.7%) (43). However, this study was criticized for mainly two shortcomings (44): (i) A mean of 2.4 and 1.6 embryos were transferred in the study group and the control group, respectively, in the fresh cycle, yielding the results difficult to interpret with today's standard of using single embryo transfer and (ii) the luteal phase support with micronized progesterone pessaries 400 mg twice daily seemed suboptimal for the POR patients and the most optimal approach would be with a combination of HCG injections and micronized progesterone pessaries 400 mg three times daily as described by Yovich previously (45) or other methods of ensuring optimal mid-luteal serum *P* levels (46).

MOST RECENT META-ANALYSES

Recently, Li et al. (47) performed a meta-analysis including 11 RCT's with a total of 663 patients. A pooled result, using fixed-effects model showed that the CPR and LBR per transfer were significantly higher in the GH group (RR 1.65, 95% CI 1.23–2.22; $p < 0.001$ and RR 1.73, 95% CI 1.25–2.40; $P < 0.001$, respectively). Moreover, the cycle cancellation rate (RR 0.65, 95% CI 0.45–0.94; $P = 0.02$) was significantly lower in GH co-treated cycles. No significant difference was seen in implantation rate (RR 1.05, 95% CI 0.56–1.99; $P = 0.87$). Although co-treatment with GH significantly increased the number of oocytes retrieved and the number of MII oocytes obtained, there was a high heterogeneity between studies regarding these two outcomes ($I^2 = 87$ and 89%, respectively) (47). The latest meta-analysis regarding the use of GH in COS was also published in 2017 (48). In that analyses all previous articles were included as well as data from the so-called LIGHT study (49). This was a multicenter, double-blind, placebo-controlled trial performed in 10 centers throughout Australia and New Zealand. Authors did not include Bologna or POSEIDON criteria for POR. A GH dose of 12IU was administered from the first day of stimulation in the intervention group. After 4 years of enrollment, the study was stopped prematurely, reporting only 130 patients randomized. The number of patients reaching an oocyte retrieval per randomized cycle was significantly higher in the GH group (62/65 [95.4%] vs. 51/65 [78.5%], OR 5.67, 95% CI 1.54–20.80), however, no differences were reported in the LBR (9/62, [14.5%] vs. 7/51, [13.7%], risk difference 0.8%, 95% CI −12.1 to 13.7%; OR 1.07, 95% CI 0.37–3.10). Unlike other studies, no statistical differences were reported between groups regarding the mean number of oocytes retrieved (5 vs. 4, rate ratio 1.25, 95% CI 0.95–1.66) and the chance of reaching embryo transfer (53/61 [86.9%] vs. 42/51 [82.4%], OR 1.42, 95% CI 0.50–4.00). No differences in embryo quality were found between groups. Results from this study should be interpreted with caution, as it was underpowered due to the few number of patients included.

CONCLUSIONS

The use of GH to enhance follicular response to gonadotropin stimulation has biological plausibility, as shown in animal and human models. The GH/IGF system plays a pivotal role in the regulation of follicular dynamics. Any experimental manipulation reducing the exposure to either GH or IGF lead to an imbalance between the primordial gonadotropin independent and gonadotropin sensitive follicular pools and a subsequent decrease in the size of the litter. However, to date, although a higher number of oocytes has been consistently reported researchers failed to show benefits in terms of LBR with the use of adjuvant GH. The use of GH is definitely "unfinished business" and future trials with bigger sample size need to be more specific as regards inclusion criteria, treatment protocol and GH dose to draw firm conclusions. Until then, it seems that some clinicians would use GH as adjuvant whereas many would not.

AUTHOR CONTRIBUTIONS

The style and concept were developed by CD, JC, TH, and PH. CD and JC produced the figure. All authors contributed to writing the manuscript, contributed with critical review, discussions regarding the final content of this review, and accepted the submission of this manuscript for publication.

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ORIGINAL RESEARCH
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Cumulative Live Birth Rates of Good and Low Prognosis Patients According to POSEIDON Criteria: A Single Center Analysis of 18,455 Treatment Cycles

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Objective: To investigate the characteristics and outcomes of low prognosis patients defined by POSEIDON criteria undergoing IVF treatment.

Design: Retrospective cohort analysis.

Setting: An IVF clinic in a public hospital.

Patients: 18,455 fresh aspirated IVF cycles with subsequently frozen embryo transfer from Jan 2014 to Jan 2017 in a single IVF clinic were included in the analysis. The low prognosis patients were categorized into 4 groups based on POSEIDON criteria: group 1: age < 35, antral follicle count (AFC) ≥ 5, number of oocytes retrieved ≤ 9 in the previous cycle; group 2: age ≥ 35, AFC$\geq$5, number of oocytes retrieved ≤ 9 in the previous cycle; group 3: age < 35, AFC < 5; group 4: age ≥ 35, AFC < 5. The non-low prognosis patients: group 5: AFC ≥ 5, previous number of oocytes retrieved > 9 oocytes; group 6: AFC ≥ 5, no previous ovarian stimulation.

Intervention(s): None.

Main Outcome Measure: The primary outcome was cumulative live birth rate (CLBR).

Result(s): Taking group 1 as reference, the CLBR from young women in group 3 (35.5%, OR 0.9, 95% CI 0.7–1.2) was slightly lower than that in group 1 (44.6%, $p = 0.615$). The CLBR in group 2 (24.5%, OR 0.6, 95% CI 0.4–0.8, $p = 0.004$) and group 4 (12.7%, OR 0.4, 95% CI 0.3–0.6, $p < 0.001$) was significant lower than that in group 1. In non-poor prognosis patients, the CLBR from young women in group 5 (53.5% OR 1.3 95% CI 0.9, 1.7, $p = 0.111$) was a slight higher than the reference group 1 while the highest CLBR was originated from the first IVF patients with good ovarian reserve in group 6 (66.9%, OR 2.0, 95% CI 1.6, 2.4).

Conclusion(s): The CLBRs and implantation rates in the young women (group 3) with diminished ovarian reserve was similar in those young women (group 1), and was significantly higher than in advanced age women with a fair ovarian reserve (group 2). Though patients in group 2 had better ovarian reserve, more oocytes and more embryos, the pregnancy outcome was inferior to that of group 3 patients with poorer ovarian reserve, fewer oocytes and fewer embryos.

Keywords: POSEIDON, cumulative live birth, implantation rate, miscarriage rate, low prognosis patient

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INTRODUCTION

Recently a novel system, the POSEIDON criteria, was developed to classify infertility patients with low prognosis undergoing assisted reproductive technology (ART) treatment (1). It is a useful system for the identification and classification of patients with impaired ovarian reserve or poor ovarian response (POR), providing guidance for the diagnosis and management of these patients (2). Four subsets have been suggested based on quantitative and qualitative parameters including, age, ovarian reserve biomarkers, and ovarian response. The new criteria, by introducing a more detailed stratification of POR, significantly reduced the heterogeneity of patients meeting the Bologna criteria (3), which may differentiate patient subsets within the POR population who could be identified and benefit from specific interventions (4). Although the POSEIDON criteria were established, along with some specific treatment recommendations proposed for the specific patient subgroups (5), there still remains insufficient evidence to support the validity of parameters used in the POSEIDON criteria, as well as the outcome assessment of different subgroups.

Among the four groups based on the POSEIDON criteria, group 1 is undoubtedly the best prognostic group considering their younger age and normal ovarian reserve, while group 4 has the worst prognosis due to the advanced age and diminished ovarian reserve. However, an interesting question is who would achieve better pregnancy outcome, the older women (group 2) with normal ovarian reserve or the young women (group 3) with diminished ovarian reserve. The cumulative live birth rate (CLBR) is considered a preferable measure of success of IVF treatment (6). Until now, there have been very few reports on the CLBRs of the four patient groups defined by the POSEIDON criteria.

The characteristics and prognosis of patients should be used to develop clinical management strategies. The objective of this study is to characterize the low prognosis patients in order to facilitate treatment decision making. In this study, the baseline characteristics and outcomes of patient groups defined by the POSEIDON criteria were analyzed, and CLBR resulting from one aspirated *in-vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycle was proposed as the primary outcome measurement for low prognosis patients undergoing IVF treatment (7).

MATERIALS AND METHODS

This retrospective study included 18,455 fresh aspirated IVF cycles with subsequent frozen embryo transfers from January 2014 to January 2017 in our center. The live birth outcome was followed up for at least 2 years until Jan 2019. The study was approved by the Ethics Committee for the Clinical Application of Human Assisted Reproductive Technology of Northwest Women's and Children's Hospital (No. 2018002). The ethics committee approved this study waived the need to obtain informed consent. All research was performed in accordance with relevant guidelines and regulations.

Inclusion Criteria

All fresh IVF/ICSI cycles and subsequently frozen embryo transfers from oocyte retrievals performed in our clinic from January 2014 to January 2017 were included in the analysis. The following cycles were excluded: (1) donated oocyte cycles (n = 28), oocyte freezing cycles (n = 8); (2) PGS/PGD cycles (n = 132); (3) cycles without live birth but with extra frozen embryos during this period (n = 337); (4) cycles of patients lost to follow-up (n = 41); (5) cycles with induced abortion (n = 18).

Patients were categorized according to POSEIDON criteria:

Low prognosis patients
Group 1 (n = 879 cycles): Age < 35, antral follicle count (AFC) ≥ 5, number of oocytes retrieved ≤ 9 in the previous cycle;
Group 2 (n = 482 cycles): Age ≥ 35, AFC≥5, previous number of oocytes retrieved ≤ 9 in the previous cycle;
Group 3 (n = 858 cycles): Age < 35, AFC < 5;
Group 4 (n = 1,306 cycles): Age ≥ 35, AFC < 5;
Non-low prognosis patients
Group 5 (n = 664 cycles): AFC ≥ 5, previous ovarian stimulation > 9 oocytes;
Group 6 (n = 13708 cycles): AFC ≥ 5, no previous ovarian stimulation.

Flow chart and data processing procedure are listed in **Figure 1**. Demographics and basal characteristics of patients are presented in **Table 1**.

Ovarian Stimulation and Oocyte Retrieval

The protocol for ovarian stimulation (OS) was determined individually according to female age, body mass index (BMI), basal follicle stimulating hormone (FSH) and antral follicle count (AFC). 94.33% of IVF patients received recombinant and/or urinary gonadotrophins (rFSH/hMG) in GnRH agonist protocol or GnRH antagonist protocol followed by IVF or ICSI. For women with diminished ovarian reserve, the mild stimulation protocol or luteal phase ovarian stimulation or natural cycle was used. Human menopausal gonadotrophin (hMG, Li Zhu, China) was added in mild ovulation protocol or Shanghai protocol according to patients' response to stimulation. Human chorionic gonadotrophin (hCG) 4,000–10,000 IU or recombinant hCG (r-hCG, MerckSerono S.p.A.) 250μg was administered when 2-3 follicles reached the size of 17 mm or higher. Thirty-six hours later, oocyte retrieval was performed using transvaginal ultrasonography-guided aspiration. The ovarian stimulation parameters of each group are listed in **Table 2**.

Embryo Transfer Policy

The oocyte processing and embryo development procedures as well as the embryo scoring system were described in our previous articles (8, 9). Grade 1–3 embryos on day 3 were considered useable embryos, and Grade 1–2 embryos were considered good-quality embryos. All fresh embryo transfers (ETs) were carried out on day 3 or day 5. In cases with sufficient number (≥ 3–4) of good-quality embryos on day 3, blastocyst transfer on day 5 would be practiced. Apart from the transferred embryos, patients' extra embryos were vitrified on day 3 or on blastocyst stage (day 5–6). Grade 1–3 cleavage stage embryos on day 3 and

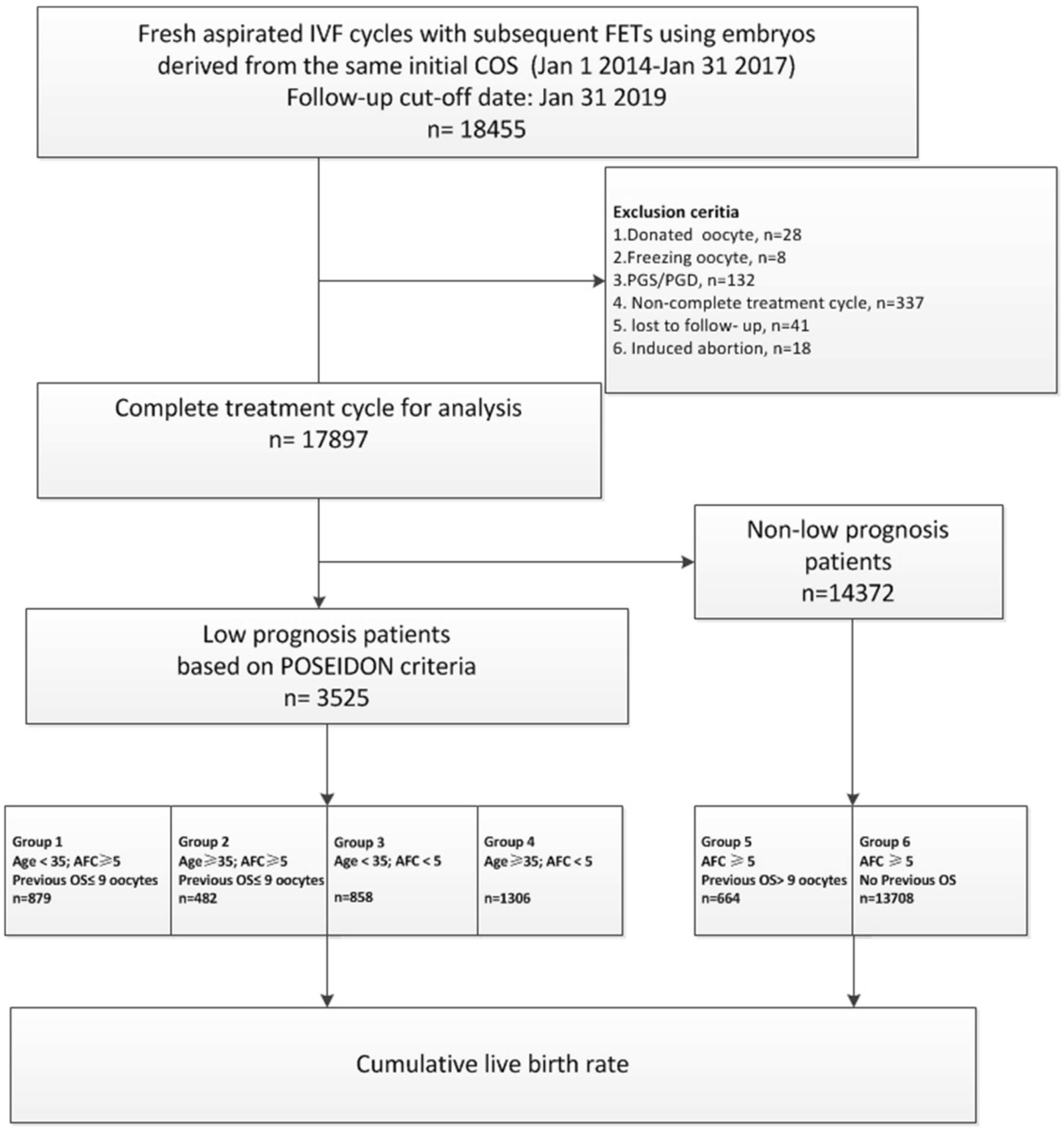

FIGURE 1 | Flow chart and data processing.

blastocysts with Gardner score above 4CC were cryopreserved (Cryo-top, open system, Kuwayama). The methods and Frozen ET procedure are detailed in previous verification study by our team (8, 9). If the implantation failed in fresh cycle, the frozen-thawed embryo transfer (FET) would be carried out using the remaining vitrified embryos or blastocysts. Patients under the age of 35 with good quality embryos were encouraged to receive a single-embryo transfer. A single embryo transfer policy was also applied for the patients who have the be abnormal uterus (e.g., scarred uterus, uterine malformation) and/ or other cases conflicted with twin pregnancy. Progesterone intramuscular injection (60 mg/day) was given for luteal phase support from the oocyte retrieval day until a negative serum beta-hCG or 8 weeks of pregnancy.

Primary Outcome Measurements and Statistical Analysis

The primary outcome was cumulative live birth (CLB) defined as at least one live birth resulting from one aspirated ART cycle in the fresh ET or in the subsequent FET in relation to the number of oocytes retrieved. The numerator of CLBR calculation was the sum of live births achieved in the FETs and live births in fresh cycles. Only the first delivery was counted in the analysis if a patient achieved multiple deliveries. The

TABLE 1 | Demographics and baseline characteristics.

Group	POSEIDON group				Non-POSEIDON group		*P*-value
	1	2	3	4	5	6	
N	879	482	858	1306	664	13708	
Year of treatment							<0.001
2014	114 (13.0%)	79 (16.4%)	184 (21.4%)	285 (21.8%)	96 (14.5%)	4154 (30.3%)	
2015	296 (33.7%)	150 (31.1%)	227 (26.5%)	344 (26.3%)	200 (30.1%)	4251 (31.0%)	
2016-2017.01	469 (53.4%)	253 (52.5%)	447 (52.1%)	677 (51.8%)	368 (55.4%)	5303 (38.7%)	
Age of female	29.9 ± 2.8	39.1 ± 3.3	30.2 ± 2.9	40.5 ± 3.5	30.9 ± 4.6	29.9 ± 4.3	<0.001
Age of female							<0.001
≤30	487 (55.4%)	0 (0.0%)	438 (51.0%)	0 (0.0%)	356 (53.6%)	8375 (61.1%)	
>30, ≤35	392 (44.6%)	63 (13.1%)	420 (49.0%)	104 (8.0%)	202 (30.4%)	3823 (27.9%)	
>35, ≤40	0 (0.0%)	262 (54.4%)	0 (0.0%)	594 (45.5%)	85 (12.8%)	1224 (8.9%)	
>40	0 (0.0%)	157 (32.6%)	0 (0.0%)	608 (46.6%)	21 (3.2%)	286 (2.1%)	
BMI of female							<0.001
≥24	228 (26.1%)	124 (26.2%)	217 (25.6%)	416 (32.3%)	180 (27.3%)	3622 (26.7%)	
≥18.5, <24	529 (60.6%)	315 (66.6%)	560 (66.0%)	813 (63.2%)	422 (64.0%)	8705 (64.1%)	
<18.5	116 (13.3%)	34 (7.2%)	72 (8.5%)	57 (4.4%)	57 (8.6%)	1248 (9.2%)	
Basal FSH (IU/ml)	7.4 ± 2.7	8.4 ± 3.5	9.3 ± 5.5	11.1 ± 7.0	6.6 ± 2.0	6.8 ± 2.6	<0.001
Type of infertility							<0.001
Primary	526 (59.8%)	123 (25.5%)	513 (59.8%)	274 (21.0%)	356 (53.6%)	7979 (58.2%)	
Secondary	353 (40.2%)	359 (74.5%)	345 (40.2%)	1032 (79.0%)	308 (46.4%)	5729 (41.8%)	
Length of infertility, year							<0.001
≤2	306 (34.9%)	193 (40.3%)	309 (36.3%)	509 (39.7%)	255 (38.6%)	5267 (38.9%)	
>2, ≤5	391 (44.6%)	134 (28.0%)	394 (46.2%)	319 (24.9%)	264 (39.9%)	5899 (43.5%)	
>5	179 (20.4%)	152 (31.7%)	149 (17.5%)	454 (35.4%)	142 (21.5%)	2385 (17.6%)	
AFC							<0.001
<4	0 (0.0%)	0 (0.0%)	521 (60.7%)	885 (67.8%)	0 (0.0%)	0 (0.0%)	
≥4, <10	484 (55.1%)	380 (78.8%)	337 (39.3%)	421 (32.2%)	148 (22.3%)	3844 (28.0%)	
≥10	395 (44.9%)	102 (21.2%)	0 (0.0%)	0 (0.0%)	516 (77.7%)	9864 (72.0%)	
Main etiology							<0.001
Pelvic-tubal factor	588 (67.3%)	342 (71.4%)	536 (62.9%)	761 (59.3%)	445 (67.5%)	8809 (64.9%)	
Ovarian factor	73 (8.4%)	36 (7.5%)	169 (19.8%)	283 (22.1%)	48 (7.3%)	1156 (8.5%)	
Male factor	94 (10.8%)	38 (7.9%)	36 (4.2%)	39 (3.0%)	82 (12.4%)	1747 (12.9%)	
Endometriosis	27 (3.1%)	2 (0.4%)	49 (5.8%)	33 (2.6%)	7 (1.1%)	198 (1.5%)	
Uterine factor	10 (1.1%)	17 (3.5%)	22 (2.6%)	83 (6.5%)	6 (0.9%)	223 (1.6%)	
Other reasons	82 (9.4%)	44 (9.2%)	40 (4.7%)	84 (6.5%)	71 (10.8%)	1438 (10.6%)	
Female smoking							0.509
No	2 (0.2%)	0 (0.0%)	2 (0.2%)	0 (0.0%)	1 (0.2%)	15 (0.1%)	
Yes	877 (99.8%)	482 (100.0%)	856 (99.8%)	1306 (100.0%)	663 (99.8%)	13693 (99.9%)	
Gravidity							<0.001
0	514 (58.7%)	116 (24.3%)	506 (59.2%)	248 (19.2%)	356 (53.8%)	7895 (57.7%)	
1	214 (24.4%)	132 (27.6%)	196 (22.9%)	306 (23.7%)	166 (25.1%)	3048 (22.3%)	
≥2	148 (16.9%)	230 (48.1%)	153 (17.9%)	737 (57.1%)	140 (21.1%)	2738 (20.0%)	
Parity							<0.001
0	820 (93.6%)	293 (61.2%)	790 (92.3%)	646 (50.0%)	588 (88.8%)	12020 (87.8%)	
1	54 (6.2%)	158 (33.0%)	65 (7.6%)	560 (43.3%)	63 (9.5%)	1511 (11.0%)	
≥2	2 (0.2%)	28 (5.8%)	1 (0.1%)	86 (6.7%)	11 (1.7%)	157 (1.1%)	
Number of oocytes retrieved in the previous cycle							NA
>10	0 (0.0%)	0 (0.0%)	4 (1.7%)	6 (1.1%)	559 (84.2%)	0 (0.0%)	
>4, ≤10	539 (61.3%)	210 (43.6%)	30 (12.4%)	57 (10.6%)	105 (15.8%)	0 (0.0%)	
≤4	340 (38.7%)	272 (56.4%)	208 (86.0%)	473 (88.2%)	0 (0.0%)	0 (0.0%)	

AFC, antral follicle count; FSH, follicle stimulating hormone; OS, ovarian stimulation.

Mean + SD / N (%), calculated using EmpowerStats (www.empowerstats.com) and R.

Kruskal Wallis Rank Test continuous variables, Chi-square tests for categorical variables, Fisher Exact for categorical variables with Expects<10.

TABLE 2 | Ovarian stimulation parameters.

Group	1	2	3	4	5	6	*P*-value
N	879	482	858	1306	664	13708	
OS protocol							<0.001
GnRH agonist	494 (56.4%)	198 (41.1%)	356 (41.7%)	428 (33.1%)	555 (84.1%)	12321 (90.1%)	
GnRH antagonist	293 (33.4%)	189 (39.2%)	274 (32.1%)	409 (31.6%)	96 (14.5%)	1215 (8.9%)	
Other	89 (10.2%)	95 (19.7%)	223 (26.1%)	456 (35.3%)	9 (1.4%)	139 (1.0%)	
Gn type							<0.001
Recombinant-FSH	325 (37.9%)	104 (22.3%)	154 (18.8%)	83 (7.1%)	343 (52.3%)	8300 (60.7%)	
Urinary -FSH	533 (62.1%)	362 (77.7%)	664 (81.2%)	1090 (92.9%)	313 (47.7%)	5372 (39.3%)	
FSH starting dose, IU							<0.001
≤150	20 (4.4%)	7 (3.1%)	30 (6.5%)	34 (5.8%)	48 (13.7%)	1682 (18.9%)	
>150, ≤300	253 (56.0%)	40 (17.9%)	201 (43.3%)	110 (18.9%)	233 (66.6%)	5749 (64.6%)	
>300	179 (39.6%)	176 (78.9%)	233 (50.2%)	439 (75.3%)	69 (19.7%)	1469 (16.5%)	
Total Gn dose IU	2999.9 ± 1100.2	3060.8 ± 1184.4	2950.7 ± 1273.0	2919.3 ± 1357.4	2783.8 ± 1039.2	2356.3 ± 971.2	<0.001
Total Gn days	10.3 ± 2.8	9.5 ± 2.9	9.4 ± 3.5	8.5 ± 3.7	11.1 ± 2.8	10.4 ± 2.2	<0.001
HMG dose	1119.7 ± 1090.8	1213.8 ± 1112.8	1195.0 ± 1133.8	1306.1 ± 1162.5	1224.7 ± 1165.9	804.6 ± 845.7	<0.001

FSH, follicle stimulating hormone; Gn, gonadotrophin; GnRH, gonadotrophin releasing hormone; HMG, human menopausal gonadotropin; OS, ovarian stimulation.
Mean + SD / N (%), calculated using EmpowerStats (www.empowerstats.com) and R.
Kruskal Wallis Rank Test for continuous variables, Chi-square testsfor categorical variables, Fisher Exact for categorical variables with Expects<10.

TABLE 3 | Oocytes and embryo parameters and CLBRs.

Group	1	2	3	4	5	6	*P*-value
N	879	482	858	1306	664	13708	
Number of Oocytes/AFC	74.00%	67.30%	145.30%	101.20%	90.50%	91.40%	<0.001
Cycles of 0 oocyte retrieved (%)	14 (1.6%)	20 (4.1%)	56 (6.5%)	157 (12.0%)	1 (0.2%)	60 (0.4%)	<0.001
Number of oocytes	7.4 ± 4.8	5.1 ± 3.7	4.3 ± 3.7	2.9 ± 2.9	12.6 ± 6.2	12.3 ± 6.7	<0.001
Number of 2PN	4.4 ± 3.3	3.3 ± 2.5	2.8 ± 2.5	2.0 ± 2.0	7.0 ± 4.3	7.4 ± 4.6	<0.001
Number of day 3 usable embryos	3.4 ± 2.8	2.6 ± 2.2	2.3 ± 2.2	1.6 ± 1.7	5.0 ± 3.7	6.2 ± 4.2	<0.001
Number of day 3 good quality embryos	1.8 ± 2.1	1.5 ± 1.8	1.3 ± 1.7	0.9 ± 1.3	2.5 ± 2.7	3.7 ± 3.3	<0.001
Cumulative live births (rate %)	392 (44.6%)	118 (24.5%)	305 (35.5%)	166 (12.7%)	355 (53.5%)	9164 (66.9%)	<0.001

AFC, antral follicle count; PN, pronucleus.
Mean + SD / N (%), calculated using EmpowerStats (www.empowerstats.com) and R.
Kruskal Wallis Rank Test for continuous variables, Chi-square tests for categorical variables, Fisher Exact for categorical variables with Expects<10.

CLBR was defined the cumulative live birth per transvaginal oocyte aspiration accordant to terminology definition (7, 10) One treatment cycle was defined as an oocyte retrieval. One complete treatment cycle referred to a treatment cycle that reached live birth or a treatment cycle that failed to reach live birth with all the embryos transferred. The cumulative live birth rate in this study was calculated based on the complete treatment cycle, so the patients ($n = 337$) of non-complete treatment were excluded.

The data processing and statistical analysis were performed using EmpowerStats software (www.empowerstats.com) and statistical software packages R. To assess the odds ratio (OR) of CLBR in different patient groups, a multiple variables regression model was established with potential confounding factors as the variables and adjusted for the year of treatment, female BMI, type of infertility, length of infertility, gravidity, parity, main etiology, OS protocol, gonadotrophin type, and FSH starting dose. Patients were enrolled for 3 years, during which IVF procedure was revised. To eliminate bias caused by this factor, the cumulative live birth rate was adjusted for the year of treatment. Female BMI, type of infertility, length of infertility, gravidity, parity, and main etiology were important factors affecting pregnancy and live birth through experience or literature. OS protocol, gonadotrophin type and FSH starting dose are the key indicators to affect the number of oocytes retrieved and ultimately the cumulative chance of live births.

RESULTS

Oocyte and Embryo Parameters

As shown in **Table 3**, the number of oocytes retrieved decreased in low prognosis patients from group 1 to group 4 ($p < 0.001$), as well as number of 2 pro-nucleus (2PN) zygotes ($p < 0.001$), number of day 3 usable embryos ($p < 0.001$) and number of good quality embryos ($p < 0.001$). Oocyte output rate

(number of oocyte retrieved/AFC x 100%) was highest in group 3 (145.3%), followed by group 4 (101.2%), group 1 (74.0%), and group 2 (67.3%).

Pregnancy Outcomes and Cumulative Live Birth Rate (CLBR)

Inconsistent with the distribution pattern of number of oocytes and embryos by group, the CLBRs in the order from highest to lowest was 44.6% in group 1($n = 879$), 35.5% in group 3($n = 858$), 24.5% in group 2 ($n = 482$) and 12.7% in group 4 ($n = 1306$). A multiple-variable regression analysis was performed with variables that may act as confounding factors described in **Tables 1**, **2**. The adjusted odds ratios (ORs) of CLBR with their 95% confidence intervals (CIs) were shown in **Table 4**. Consistent with the trend of non-adjusted results, the CLBR in group 3 (OR 0.9, 95% CI 0.7–1.2, $p = 0.615$) was slightly lower and group 5 (OR 1.3, 95% CI 0.9–1.7, $p = 0.111$) was slightly higher than CLBR in group 1 without significant statistical difference. The CLBR in group 2 was significantly lower than in group 1 (OR 0.6, 95% CI 0.4–0.8, $p = 0.004$) and CLBR in group 4 was the lowest (OR 0.4, 95% CI 0.3–0.6, $p < 0.001$) as compared to group 1. **Table 5** showed the pregnancy outcomes per fresh transfer or FET in low prognosis patients. The implantation rates in aged groups (group 2 and group 4) were significantly lower than in young groups (group 1 and group 3).

Figure 2 showed the trend chart of key events in low prognosis patients. There was a crossing of trend lines between group 2 and group 3 after embryo transfer. Patients in group 2 (age ≥35; AFC ≥ 5) had higher AFC, more oocytes retrieved, more embryos and more good quality embryos, but decreased implantation rate and CLBR. On the contrary, though patients in group 3 (age < 35; AFC < 5) had fewer oocytes and embryos, the CLBR turned out higher than that in group 2. The SWOT analysis of 4 groups of low prognosis patients defined by POSEIDON criteria is shown in **Figure 3**.

TABLE 4 | Logistic regression analysis for CLBRs.

Group	Non-adjusted OR (95% CI), *P*-value	Adjusted OR (95% CI), *P*-value
1	1	1
2	0.4 (0.3, 0.5), $p < 0.001$	0.6 (0.4, 0.8), $p = 0.004$
3	0.7 (0.6, 0.8), $p < 0.001$	0.9 (0.7, 1.2), $p = 0.615$
4	0.2 (0.1, 0.2), $p < 0.001$	0.4 (0.3, 0.6), $p < 0.001$
5	1.4 (1.2, 1.7), $p < 0.001$	1.3 (0.9, 1.7), $p = 0.111$
6	2.5 (2.2, 2.9), $p < 0.001$	2.0 (1.6, 2.4), $p < 0.001$

OR, odds ratio. OR was adjusted for the year of treatment, female BMI, type of infertility, length of infertility, gravidity, parity, main etiology, OS protocol, Gn type and FSH starting dose.

DISCUSSION

The main finding of this retrospective study in POSEIDON criteria-defined population was that the CLBR was highest in group 1, followed by group 3 and group 2, and lowest in group 4. According to our results, the CLBR from the young women with poor ovarian reserve (group 3) was slightly lower than that from young women with good ovarian reserve and previous low responder (group 1). Though the patients in group 2 (age ≥ 35; AFC ≥ 5) had better ovarian reserve, more oocytes and more embryos, the CLBR and implantation rate, on the other way round, were lower than in group 3 patients with poorer ovarian reserve, fewer oocytes and fewer embryos. This finding may facilitate the development of management strategies for low prognosis patients.

The innovative POSEIDON criteria aim at identifying and stratifying low prognosis patients into four distinct groups based on female age, AFC and ovarian response in the previous cycle (4). The patients in group 2 were characterized by good ovarian reserve and advanced age, exactly the opposite of group 3 patients with poor ovarian reserve but are at younger age. Studies (11–13) have shown that CLBR increases with the number of oocytes retrieved even in the women of advanced age (14). It was suggested the number of oocytes retrieved is a very important variable independently associated with CLBR. Patients in group 2 with a higher number of oocytes were expected to have a better prognosis than patients in group 3, because group 2 patients had more embryos to transfer. However, the CLBR and implantation rate were reversely higher in group 2 than in group 3.

Our results are consistent with previous studies (15, 16) on the association of ovarian reserve and pregnancy outcome. Chang et al. (15) found that there were lower rates of normal fertilization, cleavage, high-quality embryos, implantation, and pregnancy in older women than in younger women with

TABLE 5 | Pregnancy outcomes per transfer both fresh and frozen embryo transfer in low prognosis patients.

Group	1	2	3	4	5	6	*P*-value
Transfer cycle (fresh ET + FET)	1,126	602	781	976	1,199	18,862	
Number of embryos transferred	1.8 ± 0.5	1.9 ± 0.5	1.7 ± 0.5	1.7 ± 0.6	1.9 ± 0.5	1.7 ± 0.5	<0.001
Implantation rate	34.40%	21.26%	40.45%	19.19%	29.49%	48.24%	<0.001
Pregnancy loss rate/ transfer	9.68%	10.80%	9.99%	9.22%	9.84%	9.47%	0.896
Miscarriage in first trimester/ transfer	7.46%	9.14%	6.91%	8.20%	6.84%	6.56%	0.059

ET, embryo transfer; FET, Frozen embryo transfer.
Kruskal Wallis Rank Test for continuous variables, Chi-square tests for categorical variables, Fisher Exact for categorical variables with Expects<10.

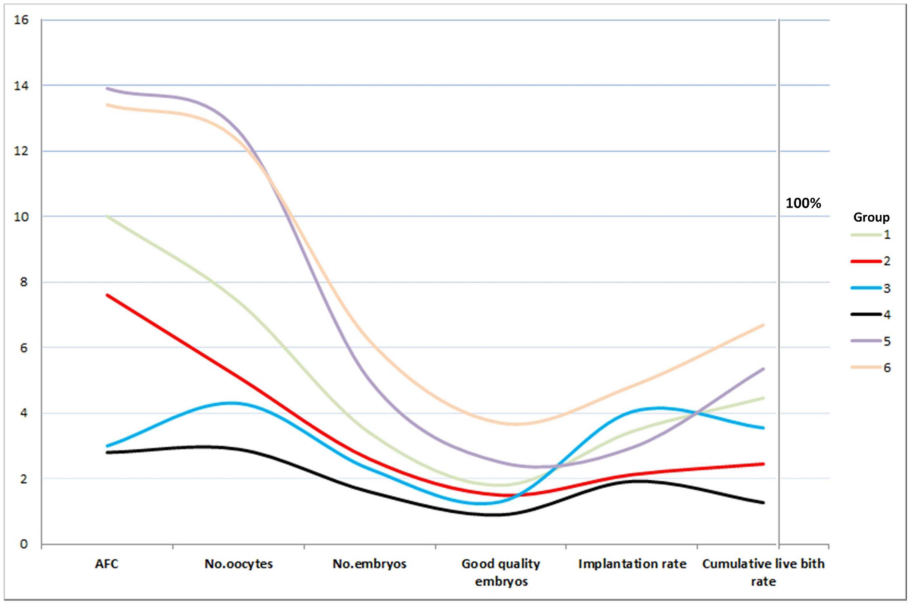

FIGURE 2 | Trend chart of key events in low prognosis patients. There was a crossing of trend lines between group 2 (red) and group 3 (blue) after embryo transfer. X axis represents the average number of AFC, number of oocytes, number embryos, number of good quality embryos, rate of implantation and rate of cumulative live birth. The Y axis on the left represents the number of the first four variables (*n*) and the Y axis on the right represents the rate of last two variables (%).

FIGURE 3 | SWOT analysis of 4 groups of low prognosis patients defined by POSEIDON criteria.

diminished ovarian reserve. The primary reason was the adverse impact of aging oocyte on the pregnancy outcome (low implantation rate and high pregnancy loss rate) due to chromosomal abnormalities (17) and cytoplasmic dysfunction (18). The decline in fertility with aging involves both quantity and quality of oocyte. Implantation and miscarriage are related to the quality of oocytes but not necessarily the ovarian reserve (16). The fair ovarian reserve in group 2 would increase the possibility of obtaining more oocytes and embryos to transfer, but at the same time, the higher aneuploidy rate would lead to low implantation rate and high miscarriage rate.

In terms of the management of patients in group 2, more attention should be paid to develop strategies of improving the oocyte quality rather than oocyte quantity or embryo quantity. Because more embryos are achieved on day 3 in group 2, culturing embryos to blastocyst stage for transfer is a good option. Day 5–6 embryos have lower rate of segmental aneuploidy (19) and higher viability for implantation (20) than day 2–3 embryos. Preimplantation genetic testing for aneuploidies (PGT-A) are also beneficial for advanced women to select an euploid embryo to transfer (21). An optimal ovarian stimulation regimen to improve the quality of oocytes (22, 23) could be an alternative option. Supplements such as dehydroepiandrosterone were tried to improve follicle development (24), though there is insufficient evidence to support their use in these patients.

The patients in group 3 (age $<$ 35; AFC $<$ 5) had a poor ovarian reserve, who were expected poor responders with poor pregnancy outcome. Interestingly, the oocytes output rate (145.3% oocytes retrieved per AFC) in group 3 was significantly higher than in the other groups, though FSH starting dose was not significantly increased (**Table 2**). This suggested the response of antral follicles to gonadotropin may have reached the limit of its ability, therefore there will be no additional benefit in oocyte number to further increase daily gonadotrophin doses (25). Evaluating ovarian sensitivity to FSH is a key element to improve IVF success rates in these low prognosis patients and open new treatment perspectives (26). The high oocytes output rate per AFC in group 3 also supported the reported recommendation of maximum daily dose of 300 IU rFSH (5). For the patients in group 3, more efforts should be focused on increasing the number of oocytes, as the clinical pregnancy outcome is reassured once oocytes are acquired.

The patients in group 5 were the non-low prognosis patients who had good ovarian response (more than 9 oocytes in previous retrieval), however most of these patients failed to live birth in previous IVF cycle. Comparing with another non-low prognosis patients with first IVF treatment (group 6), though the patients in group 5 have more AFC and number of oocytes retrieved, the embryo development and pregnancy outcome were inferior to that in group 6. Therefore, the failure in first IVF patients with good ovarian response may be the poor prognosis for subsequently IVF treatment.

Pelvic-tubal factor is the most common cause of infertility, accounting for about 10.8–78.3% of infertile women in China (27, 28). Tubal factor mainly involves tubal occlusion and peritoneal pathology causing adhesions, which was diagnosed by hysterosalpingography and laparoscopy. The prevalent cause of tubal factor infertility was attributed to pelvic inflammatory disease (PID), salpingitis and endometriosis (29). In the patient groups of this study, tubal factor is the main infertility etiology, ranging from 62.9 to 71.4%, which was higher than that in infertility women of other countries and regions. Therefore, it should be careful to interpret the wider implications of the findings.

Limitations are related to the retrospective nature of the study and the fact that the data was from a single center also weakens the universality of our observations. Other potential limitations could be that non-GnRH analog protocol was used in some women with diminished ovarian reserve rather than in those with normal ovarian reserve. The fresh cycles that failed to yield any oocyte were not included in this study, for example, cycles with cancellation of ovarian stimulation.

The results of this study may provide new insights for the development of management strategies for low prognosis patients. A SWOT analysis was performed to help the management for poor prognosis patients in clinic, which was drawn from the POSEIDON reports (1, 2, 4, 5) and the data in this study. The responsiveness of antral follicles to gonadotrophin was extremely higher in group 3 than in the other groups. Considering the gratifying CLBR outcome in group 3, in order to increase oocyte yield, we suggest to try more ovarian stimulations but not harder ovarian stimulation through excessive daily gonadotrophin dose. Though the patients in group 2 have more ovarian reserve as well as more oocytes and embryos, the CLBR was lower than expected. The management strategy for group 2 should be improving the live birth rate rather than increasing number of oocytes retrieved.

CONCLUSION

In conclusion, inconsistent with the distribution pattern of oocyte quantity and embryo quantity by patient group, the CLBRs in the order from highest to lowest were in group 1 (young women with good ovarian reserve), group 3 (young women with poor ovarian reserve), group 2 (women at advanced age with good ovarian reserve), and at last group 4 (women at advanced age with poor ovarian reserve.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The study was approved by the Ethics Committee for the Clinical Application of Human Assisted Reproductive Technology of Northwest Women's and Children's Hospital (No. 2018002). The ethics committee that approved this study waived the need to obtain informed consent. All of the research was performed in accordance with the relevant guidelines and regulations.

AUTHOR CONTRIBUTIONS

WS and JS conceived and designed the study. WS and WZ drafted and revised the manuscript. WS, ZZ, and WZ analyzed and interpreted the data. LT, ZZ, and HZ collected and cleared the data. All authors have read and approved the final version of the manuscript.

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REVIEW
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The Role of Growth Hormone on Ovarian Functioning and Ovarian Angiogenesis

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Although not yet well-understood, today it is clear that Growth Hormone (GH) exerts a relevant role in the regulation of ovulation and fertility; in fact, fertility is lower in women with GH deficiency (GHD), and GH receptors (GHR) and GH mRNA have been found in the ovary since the onset of follicular development in humans. However, despite the strong evidence of GH in the regulation of fertility, many aspects of GH actions at this level are still not well-established, and it is likely that some controversial data depend on the species analyzed, the dose of the hormone and the duration of use of GH. Folliculogenesis, ovulation, and corpus luteum formation and maintenance are processes that are critically dependent on angiogenesis. In the ovary, new blood vessel formation facilitates oxygen, nutrients, and hormone substrate delivery, and also secures transfer of different hormones to targeted cells. Some growth factors and hormones overlap their actions in order to control the angiogenic process for fertility. However, we still know very little about the factors that play a critical role in the vascular changes that occur during folliculogenesis or luteal regression. To promote and maintain the production of VEGF-A in granulosa cells, the effects of local factors such as IGF-I and steroids are needed; that VEGF-A-inducing effect cannot be induced by luteinizing hormone (LH) or chorionic gonadotropin (CG) alone. As a result of the influences that GH exerts on the hypothalamic-pituitary-gonadal axis, facilitating the release of gonadotropins, and given the relationship between GH and local ovarian factors such as VEGF-A, FGF-2, IGF-1, or production of sex steroids, we assume that GH has to be a necessary factor in ovarian angiogenesis, as it happens in other vascular beds. In this review we will discuss the actions of GH in the ovary, most of them likely due to the local production of the hormone and its mediators.

Keywords: growth hormone, IGF-1, leptin, kisspeptin, GnRH, puberty, menopause, ovarian angiogenesis

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INTRODUCTION

Classically, since its discovery, isolation and administration to a pituitary dwarf (1), the pituitary growth hormone (GH) has been considered a metabolic hormone that, in addition, has specific effects on growth until puberty ends. This concept remains widely valid, despite the many and very important physiological roles that GH plays in the human body, now well-known, in virtually all tissues and organs (2). A schematic representation of these multiple functions of GH can be seen in Caicedo et al. (3), Figure 1 in this reference. Moreover, for years, we have known that apart from

the endocrine GH expressed and released from the pituitary gland, there are expressions of the hormone in many cells and tissues, where it plays autocrine/paracrine roles (4), the meaning of which is still little understood in many cases, although its importance is beyond doubt in situations such as for example, the induction of cell proliferation or survival, or inducing the expression of glucose transporters which allow the uptake of glucose needed for the production of energy by cells. In addition, after interacting with its membrane receptor, extracellular GH is translocated to the nucleus (5), where the hormone interacts with its receptor (GHR), and its binding protein GHBP (the extracellular domain of GHR), inducing changes in transcriptional activity (5, 6). These findings contradicted the classical concept about the fact that protein hormones only acted on their receptors in the cell membrane. More recent studies introduce more complexity about the mechanisms of action of GH at the cellular level. It has been demonstrated, in pigs, that GH administration induces the translocation of GHR to cell nuclei *in vivo*, which may indicate that the nuclear GHR exerts functions that we do not yet know (7), although presumably, it acts there as a transcriptional factor.

In any case, the supply of GH to cells or tissues needs an adequate blood flow, and the hormone plays a very important role in the formation of new blood vessels or the recovery of damaged vasculature (3).

In the case of gonadal function in females, it seems to be of interest that, to our knowledge, the first description of the existence of a strong immunoreactivity for the GH receptor at the nuclear level, was described in 1990 in rat oocytes (8) (**Figure 1**). The same study found a very large distribution of the GHR/GHBP in practically all the reproductive system of the rats analyzed, suggesting that GH could play important and direct actions on reproduction, but also in the normality and integrity of the endometrial vascular system (8). Interestingly, these authors also found that this GHR/GHBP immunoreactivity was clearly higher in the ovarian granulosa cells from 10 days old rats than in granulosa cells from adult animals. This could be related to the fact that the pituitary secretion of GH gradually decreases along aging (9), but also with the fact that vasculature suffers progressive damage along life (3), with the subsequent decrease of blood supply to the ovary.

In this review, we will analyze the role played by GH for a normal ovarian function, and also the role of ovarian angiogenesis in ovarian functioning, as well as the effects of aging on both processes.

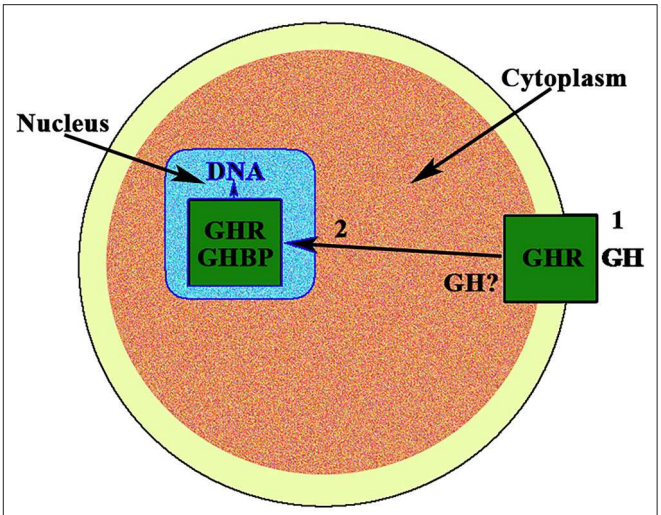

FIGURE 1 | GH receptor in the nucleus of the oocyte. In the nucleus of the oocyte, the GH receptor (GHR) and the carrier protein GHBP have been found. This means that GH, after interacting with its membrane receptor (1), has allowed the internalization of both, GH and GHR, (2) and then the receptor is translocated to the cell nucleus where it would act as a gene transcription factor. The possibility exists that the own GH is expressed in the oocyte.

OVARIAN FUNCTIONING

Ovarian Cycle

The ovarian gland suffers continuous physiological and morphological changes from embryogenesis until menopause and during the physiological ovarian cycle, most of them clearly related with the intervention of GH, apart from pituitary gonadotropins. As is logical, these changes must be accompanied by changes in the blood supply to ensure adequate supply, not only of nutrients but also of hormones and factors involved in these changes. This, in turn, means that the number, size, distribution, and functionality (permeability) of the blood vessels must also change considerably throughout the life of this gland, not only at the beginning of puberty but also daily, in each menstrual cycle, and during the reproductive life of a woman, until menopause. Therefore, ovarian angiogenesis is also a critically regulated process, both for ovulation and for development and function of the corpus luteum (CL).

In fact, once the puberty begins, folliculogenesis, ovulation, and CL formation and maintenance are processes that are critically dependent on angiogenesis. Irrigation of the ovary comes from a direct branch of the abdominal aorta, the ovarian artery, although this artery is anastomosed with a second source of ovarian irrigation, the ovarian branches of the uterine arteries, coming from the internal iliac arteries. Although these main sources of blood supply to the ovary do not change physiologically, an intricate network of pre-existing, initially non-functional, arterioles and newly formed capillaries arise from them, and continually change to support the continuous changes that occur in follicular development, ovulation, luteogenesis, and luteolysis. This is in a clear contrast with that occurring in other organs and tissues, in whom, physiologically, the vascular system does not suffer significant changes (10).

In addition, the ovary is an endocrine gland from which sex steroids are released into the blood under the stimulation of pituitary gonadotropins (Gns), to perform very important functions in the female body, but also to regulate in the ovary the sequence of processes that lead to ovulation and luteogenesis. This concept is schematically represented in **Figure 2**.

Growth Hormone and Ovarian Functioning

As described in the Introduction, the presence of GHR in rat oocytes, and also in practically the entire reproductive system

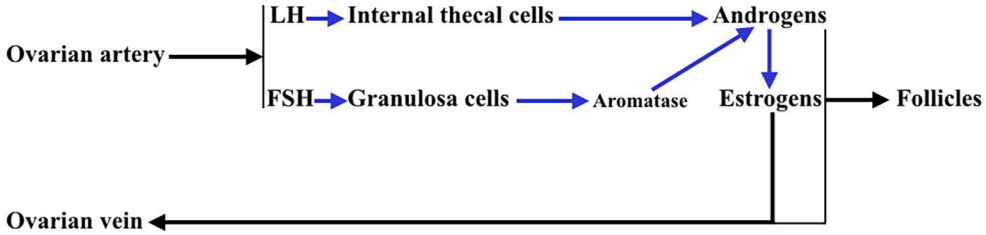

FIGURE 2 | Interactions between the theca cells of the internal layer and the granulosa cells in a growing follicle. Under the stimulus of pituitary LH, the theca cells produce androgens that reach the granulosa cells and in these, under the control of pituitary FSH, that activates aromatase, they are aromatized to estrogens (mainly estradiol). These estrogens are released into the systemic circulation and follicular fluid. Likewise, a small part of the androgens produced in the thecal cells pass into the systemic circulation.

of these animals was found almost 30 years ago (8). This was the first indication that GH played a very important role in the reproductive system of this species, something posteriorly verified in other animal models (11). Later studies showed that there is a genetic expression of GHR in cumulus cells, mature oocytes and preimplantation human embryos, in which there is a high expression of GHR from the 4-day morula onwards (12). This study led to the conclusion that, in humans, GH plays a role in the maturation of the oocyte and embryogenesis, from its early stages. GH and GHR have been found in human ovaries from fetuses and adults (13), where they play a very important autocrine/paracrine role.

Although most of the GH effects on the ovarian function are exerted by the hormone locally produced in the ovaries, systemic GH released by the pituitary gland or exogenously administered, also plays an important role in the normal function of the female gonad and reproduction, as previously reviewed by Hull and Harvey (14). Gonadal steroids participate in the hypothalamic regulation of pituitary GH secretion (9); GH pulse amplitude increases in hypogonadal prepubertal girls with Turner's syndrome when they receive E2 (15). In turn, GH participates in the regulation of puberty and fertility through changes in Gns secretion, directly or via IGF-1 (16, 17). Hypothalamic neurons producing the gonadotropin-releasing hormone (GnRH) express IGF-1 receptors, therefore Gns secretion may be regulated, at least partially, by the GH/IGF-1 axis (16, 18, 19). IGF-1 has been shown to stimulate GnRH promoter activity *in vitro* (20, 21). IGF-1 has been found in the brain, and there its expression is stimulated by GH, at least in human neural stem cells (22). IGF-1 is able, in rats, to stimulate the biosynthetic activity of pituitary gonadotrophs *in vitro* (18), and it has been shown that this peptide acts directly on GnRH neurons for the regulation of puberty in rats (19), although it can also act on kisspeptin neurons, which play a key role in regulating the activity of GnRH neurons [for review, see (19)]. A very recent study reveals that GH directly exerts effects on kisspeptin neurons located in the anteroventral periventricular and rostral periventricular nuclei via the signal transducer and activator of transcription 5 (STAT5), while the hormone lacks any effect on kisspeptin neurons located at the arcuate nucleus (23). These data agree with clinical data indicating that an appropriate secretion of GH is needed for sexual maturation and maintenance of reproductive functions, while GH deficiency may affect the beginning of the puberty and can produce infertility. In humans, the interaction GH–GHR in the ovary promotes the synthesis of sex steroids and induces gametogenesis, inhibits follicular apoptosis, and upregulates ovarian receptors for LH (14, 24, 25). These concepts are schematized in **Figure 3**. GH replacement therapy restores normal ovarian function in women with GH deficiency (26), in which the onset of puberty is delayed and reproductive function altered, and in infertile eugonadal women with GH deficiency in whom GH treatment restores fertility with successful pregnancies (27).

Growth Hormone and Puberty in Females

For years, it has been known that the onset of puberty is characterized by a change in the secretion pattern of pituitary gonadotropins. During childhood both gonadotropins circulate in low levels, and FSH secretion clearly predominates over LH secretion, whereas when puberty begins, not only do the plasma levels of both gonadotropins increase, but this pattern is also inverted. First, only during the night is the secretion of LH higher than that of FSH, and then throughout the day the menarche appears and menstrual cycles begin (28, 29). These pubertal changes occur as a consequence of the activation of the GnRH pulse generator, previously practically quiescent, produced by neurotransmitters acting on GnRH neurons (29) and the stimulatory effect of hypothalamic kisspeptin on them (30). The question is: why does this activation of the GnRH pulse generator usually occur at a certain age, highly variable among girls, during development? It is well-known that the onset of the puberty shows great changes among different ethnic populations throughout the world, which indicates that there is a genetic control on the timing of puberty (31). Although most likely the onset of puberty is polygenic, in a high number of girls a study identified the Single Nucleotide Polymorphism (SNP) rs314276 in the intron 2 of *LIN28B* gene, located on chromosome 6, as the first genetic marker associated with menarche (32), although other genes also contribute to the physiology of this important event in the development (33, 34). **Figure 4A** shows the changes in gonadotropins and GH secretion from childhood to old age.

Besides its genetic determinant, the onset of the puberty is conditioned by the nutritional status of the individual, something

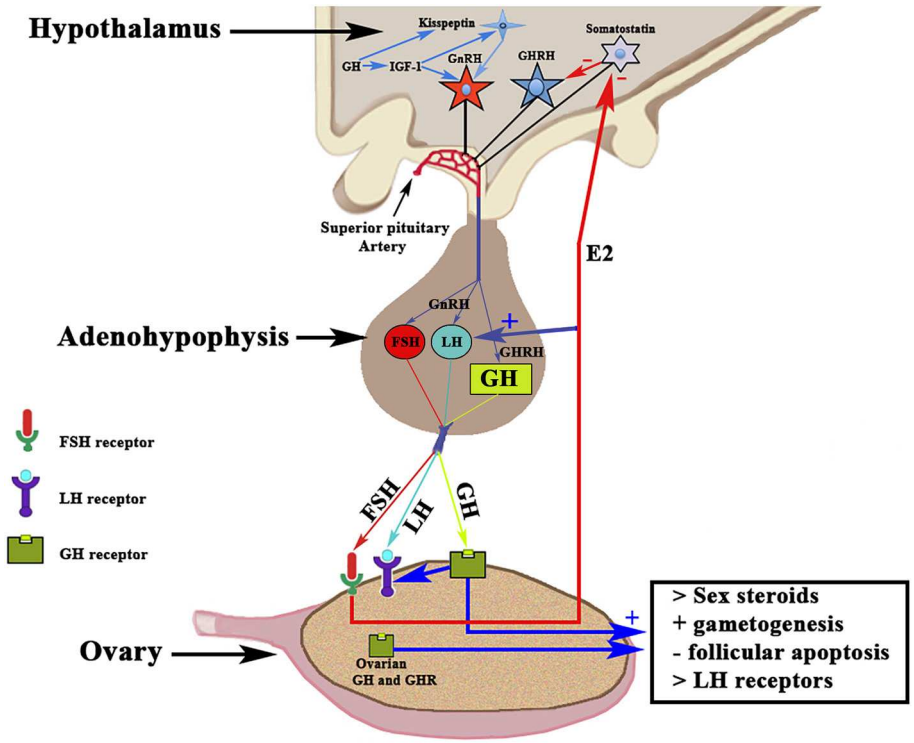

FIGURE 3 | Schematic representation of the Hypothalamic-Pituitary-Ovarian axis and the effects of GH on ovarian functioning. The activation of the GnRH pulse generator leads to the release of GnRH in the portal blood from which it reaches the gonadotropic cells in which it induces the release of FSH and LH (in proportions and amounts variable throughout a menstrual cycle) in the systemic circulation. Both gonadotropins interact with their ovarian receptors, triggering the previously described effects. The release of pituitary GH is induced by hypothalamic GHRH, which is negatively regulated by somatostatin. Systemic GH interacts with its ovarian receptor GHR and induces the positive regulation of LH receptors. The activation of GHR induces, throughout its effects on thecal cells, an increase in the production of sex steroids, mainly estradiol (E2), which in addition to its actions at the ovarian level, is released into the general circulation. Estradiol increases the pituitary release of LH, and also acts by inhibiting the hypothalamic release of somatostatin, which allows GHRH to be released into portal blood and stimulate pituitary synthesis and release of GH. GH and its GHR also are produced in the ovary; therefore, the ovarian actions of GH may also depend on the GH–GHR interaction. GH acts on the oocyte and the survival of the ovarian follicles. GH is also produced in the brain, where it stimulates the synthesis of IGF-1 that activates the GnRH pulse generator. In addition, both brain GH and IGF-1 stimulate kisspeptin neurons to release kisspeptin, a key factor in the activation of the GnRH pulse generator. +, stimulation; –, inhibition.

logical as a minimum availability of available energy is necessary to face the beginning of a reproductive stage. In fact, increased fat mass has been associated with precocious puberty (35, 36), while undernutrition or weight loss lead to delayed menarche or amenorrhea produced by hypogonadotropic hypogonadism (37). In line with this, leptin, a hormone produced by the adipose tissue (38), to regulate body fat mass by inhibiting food intake by stimulating the satiety center, is related to the onset of puberty. The reason is that increased fat mass would lead to increased leptin production, and since obesity induces precocious puberty, leptin has been considered as a metabolic signal to the reproductive system (39). More recent studies indicate that high levels of leptin in prepubertal girls are clearly associated with menarche at younger ages (40). The permissive role of leptin in the onset of puberty has been suggested to be mediated by kisspeptin neurons, given that alterations of the *KISS1* gene and the kisspeptin receptor have been associated with leptin-deficient production (41). Moreover, reproductive alterations can be recovered by leptin administration, both in humans and animals. Therefore, there seems to be a leptin-kisspeptin-GnRH pathway that carries metabolic information to the centers that regulate reproductive function (42). These concepts are shown in **Figure 4B**.

Once we have briefly analyzed how puberty starts in girls, it is appropriate to assess whether GH plays a role in this process. The pituitary secretion of GH increases sharply as puberty approaches; consequently, the rate of growth velocity also increases to more than double or triple the values observed throughout childhood (2). This apparent relationship between increased GH secretion and the onset of puberty led to the assumption that GH could act as a co-gonadotropin that increases the effects of FSH and LH on the production of ovarian sex steroids (43). In this line, GH-deficiency has been identified as the only cause of primary amenorrhea in three adolescent women in whom the secretion of gonadotropins was normal, suggesting that GH would play a complementary role to gonadotropins for the onset of menarche (44). As stated above, GH-deficiency negatively affects ovarian function in humans delaying sexual maturation (26) and fertility (27), a situation that is reversed with GH replacement therapy.

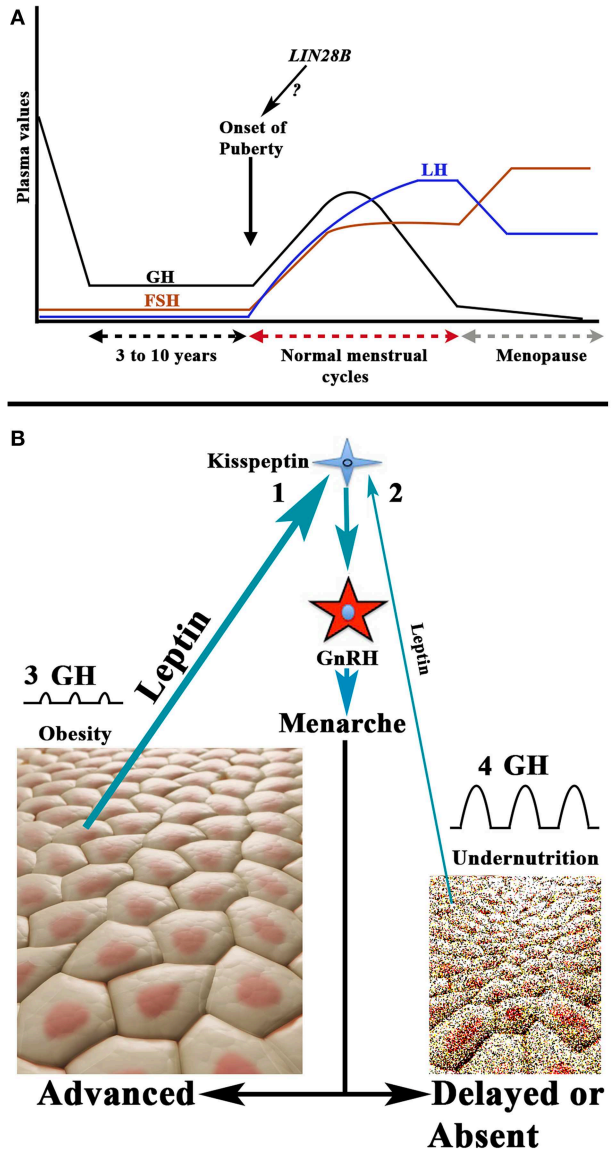

FIGURE 4 | Schematic representation of the secretion of gonadotropins and GH from childhood to aging, and the relationship between nutritional status and the onset of puberty. **(A)** Plasma GH, LH, and FSH values from childhood to aging in a normal woman. Note that during childhood the FSH levels, although low, are permanently higher than plasma levels of LH. The onset of puberty is characterized by a nocturnal increase of LH on the plasma values of FSH; then LH is always at higher values than that of FSH (with the exception of the first days of the follicular phase and the last days of the luteal phase during a menstrual cycle). At menopause, the secretion of both gonadotropins increases considerably, and the release of FSH is markedly higher than that of LH. The evolution of the secretion of GH follows a pattern similar to that of LH up to 18–20 years. From this age it decreases gradually until it is practically undetectable in old age. Curiously, the highest secretion of GH occurs when puberty begins. As shown in the figure the possibility exists that the *LIN28* gene activation may be responsible for the onset of puberty. **(B)** There is a clear relationship between the body fat mass and the activation of the GnRH pulse generator. Adipocytes release leptin which, among other actions, stimulates the production of kisspeptin who, in turn, activates the production and secretion of GnRH. This leads to menarche. Therefore, the greater amount of body fat, the higher secretion of leptin (1) and the lower age of onset of the *(Continued)*

FIGURE 4 | puberty, while undernutrition leads to decreased leptin production (2) and this leads to delayed or absent puberty onset. In these opposite situations, the rate of secretion of GH does not take parallel to the onset of puberty. In obesity, the secretion of GH is considerably reduced or absent (3), whereas in girls with low fat mass the secretion of GH is markedly increased (4). This is probably due to the fact that the increase in the fat mass produces an increase in the hepatic production of IGF-1, which induces the hypothalamic release of somatostatin, but also directly inhibits the pituitary release of GH, while undernutrition prevents the synthesis of IGF-1 in the liver, therefore leading to an increase in GH secretion.

It has been postulated that the increase of GH secretion when puberty begins depends on the activation of the GnRH pulse generator (43); the consequent increase in the secretion of LH would stimulate the production and secretion of ovarian sex steroids, which, in turn, increase noradrenergic pathways that acting on alpha-2 receptors block somatostatin secretion and allow the hypothalamic release of GHRH (9); this would lead to an increase in the synthesis and secretion of pituitary GH and would explain the increase in plasma levels of this hormone and the rate of growth during puberty (**Figure 4A**). Although this possibility is real, the fact that GH and IGF-1 stimulate, in rats, the activity of the GnRH promoter *in vitro* (18, 19) and kisspeptin neurons (23), may indicate that the activation of the GnRH pulse generator is an event that follows the increased secretion of GH/IGF-1 that occurs when pubertal maturation begins. Another possibility is that both processes occur in parallel and, physiologically, each one of them provides positive feedback to the other. It would be of interest to investigate whether GH might participate in the regulation of the expression of *LIN28B* gene or other genes involved in the onset of puberty.

As described above, the onset of puberty in girls is also dependent on the nutritional status. A recent meta-analysis indicates that obesity leads to early onset of puberty (45). This corroborates other studies that relate the increase in fat mass and early menarche (35, 36, 46–50). However, obesity leads to a reduction or absence of GH secretion (2, 37, 50) (**Figure 4B**), suggesting that GH does not play a role in the onset of puberty. Despite the lack of a significant secretion of GH, although a residual secretion of the hormone in this situation cannot be discarded, the growth rate is normal or even higher in obese girls than in girls with normal fat mass (2). This can be explained by the higher levels of IGF-1 in obesity, which is the real hormone responsible for growth (2), just as early puberty in these cases can be explained by the increase in IGF-1 itself (17) and the high levels of leptin produced by the excessive fat tissue (40) (**Figure 4B**).

In summary, the onset of puberty in girls is a very complex process in which many factors participate. Among them, genetic factors, nutritional status, environmental factors, ethnicity, but also hormonal factors such as GH/IGF-I and leptin. GH acts in conjunction with gonadotropins and perhaps is an inducer of the activation of the GnRH pulse generator.

Growth Hormone and Menstrual Cycles

GH is a hormone that plays a very important role in the course of the processes that during a normal menstrual cycle culminate

in the maturation and release of the oocytes; this function can be performed by the systemic hormone or the one produced in the ovary itself, but it can also depend on the ovarian IGF-1 induced by GH, or independently produced in this gland. The attribution of a specific effect to one of these hormones or to the GH/IGF-1 system is generally difficult to be established, as most of the data on the effects of GH and IGF-1 at the ovarian level come from cultured cells or studies in animals, and the effects seem to be different depending on the species. For example, it has recently been shown in ovarian sheep cultures, that IGF-1 is present in all stages of follicular development (51). In this study, IGF-1 was found in oocytes and GCs of antral follicles, acting synergistically with FSH to stimulate oocyte growth, increasing the number of fully developed oocytes and increasing the immunoreactivity of the LH receptor in GCs (51). This effect of IGF-1 seems to be dependent on GH, at least in bovines (52). Data from studies in cultured human GCs indicate that GH stimulates the production of IGF-2, which suggest that IGF-2 may be also a mediator of GH effects in follicles acting through the receptor for IGF-1 (53).

In any case, data from studies in women indicate that the GH/IGF-1 system plays a very important role during the human menstrual cycle.

To produce a viable normal embryo, a sequence of perfectly linked processes must occur in the post-menarchal ovary. These are: steroidogenesis, folliculogenesis, oocyte maturation, and luteogenesis. After menarche, oocytes secrete a number of factors that act on the surrounding GCs to regulate the development and function of these cells (54, 55); this allows the primary follicles to develop into secondary follicles and then grow to the preantral and antral stages, under the control of pituitary Gns, but also GH, as *in vivo* studies demonstrated that this hormone seems to be necessary for achieving an optimal maturation and survival of developing follicles (14, 56, 57), and increases ovarian weight in some species. In contrast, the absence of effects of GH, as occurs in knockout mice for GHR, leads to these animals to have few primary, secondary, preantral and antral follicles, and increased follicular atresia (25, 58).

A normal menstrual cycle begins with a predominant secretion of FSH over LH. In this situation, growing preantral follicles TCs, stimulated by LH, produce androgens, mainly androstenedione, which reach GCs where they are converted in E2 by the action of an FSH-dependent aromatase (**Figure 2**). Years ago it was found that in 24 normal patients, with normo-ovulatory cycles, short-term GH administration exerted a synergistic effect on the FSH-induced follicular steroidogenesis, in terms of increased E2 production during the early follicular phase, significantly higher than that induced by FSH injection without GH administration, while GH alone was unable to induce any steroidogenic response, as happened when saline was given to these women (59). This GH effect might be attributed to an action of the hormone on the activity or expression of aromatase (**Figure 5A**). However, it has been demonstrated that GH inhibits FSH-induced aromatase activity via an IGF-1 independent pathway, therefore inhibiting E2 synthesis in rat GCs, while, as demonstrated many years ago, IGF-1 stimulates aromatase activity (60). It has been shown that GH increases IGF-1 and its receptor, and receptors for FSH in rat GCs via phosphorylation of Signal Transducers and Activators of Transcription (STATS), while the family of bone morphogenetic proteins (BMP) inhibits IGF-1 effects on FSH-induced E2 production by suppressing the expression of the ovarian GH/IGF-1 (61) (**Figure 5B**); curiously, the GH/IGF-1 system down-regulates the expression of BMP receptors, and GH upregulates inhibitors of BMP signaling, therefore negatively affecting BMP signaling pathways (61). Regarding P4 synthesis in GCs, it is induced by FSH and GH enhances this effect through induction of steroidogenic acute regulatory protein (StAR), cytochrome P450 and 3ß-hydroxysteroid dehydrogenase, but this is blocked by BMP (**Figure 5C**). Therefore, and since the expression of BMP members changes throughout a menstrual cycle, it seems that the relationship between GH/IGF-1 and BMP signal intensities, and that the type of them, plays a key role in the regulation of Gns-dependent steroidogenesis in follicles. Moreover, BMP also participate in the regulation of the hypothalamic secretion of GnRH and ovarian sensitivity to Gns (62). These effects of BMP, particularly BMP-15, have been also seen in humans (63, 64).

In humans, as in many other species, GH seems to play a direct role in the nuclear maturation of oocytes (12, 65). In human oocytes, the receptor for GH has been detected in the membrane, in cumulus cells (66) and in the nucleus in mature ovaries (13), a fact that confirms that GH has to act at this level improving nuclear maturation and the expansion of cumulus cells, as has been demonstrated in primates (67), and also improving the cytoplasmic maturation of mature oocytes (68).

Once ovulation occurred there is the need of maintaining the corpus luteum and the secretion of the needed amount of P4 from it if pregnancy occurs, until the placenta begins to produce its own P4. This allows implantation and avoids abortion. It has been shown that leptin acts synergistically with GH and IGF-1 in the luteinization of GCs, which begins before follicular disruption and ovulation (69). In this context, GH also seems to play a very important role as proliferative and antiapoptotic factor. GH together with FSH stimulates the proliferation of luteinized GCs (70) and avoids the apoptosis of these cells (**Figure 6**). The detection of the GHR in human luteal cells (8) supports the postulate that GH exerts a role in the important functions of the corpus luteum.

In addition to the important actions that the GH/IGF-1 system exerts on the ovaries, GH also facilitates the development of the most appropriate maternal environment, acting in the uterus very early in gestation. Both GH and its receptor are expressed in the uterus of pregnant and non-pregnant women [for review, see (2)]. GH induces uterine growth, thus facilitating implantation. In fact, pregnancy maintenance requires significant uterine hypertrophy. Clinical studies carried out in women with thin endometrium indicate that patients treated with GH developed greater endometrium thickness on day 3, higher implantation rates and greater clinical pregnancy rates than the untreated control patients (71). These effects have been attributed to the fact that the hormone, via JAK2/STAT5 pathway, promotes the proliferation of endometrial cells and increases uterine vascularization (through the regulation of VEGF-A expression); these GH actions could be mediated by the autocrine IGF-1 or

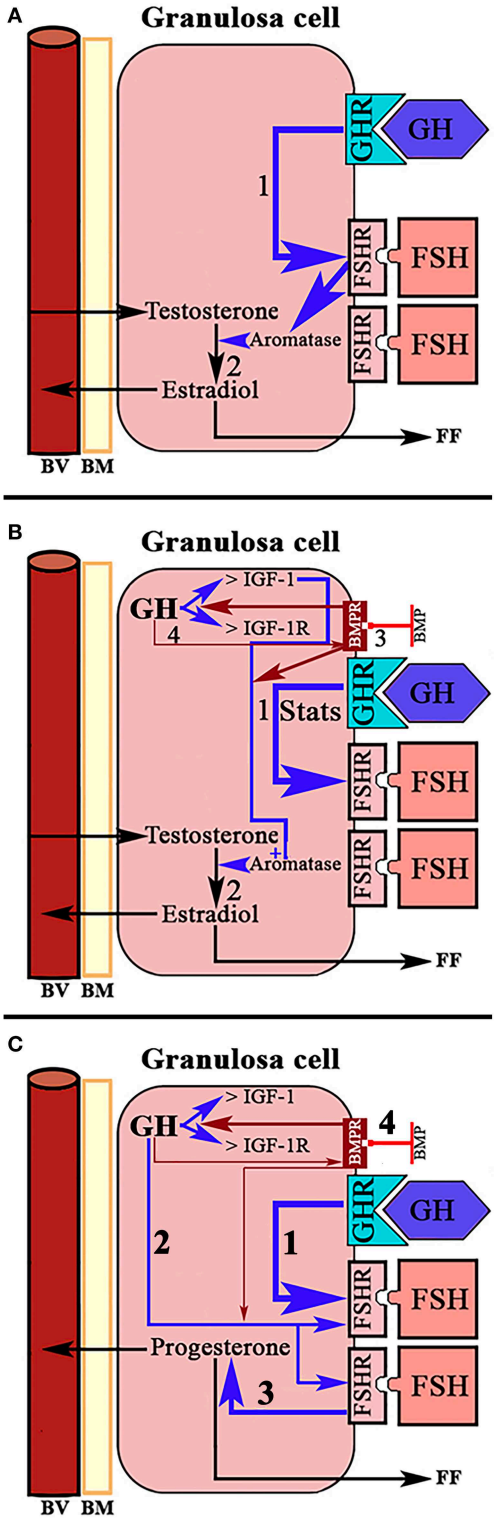

FIGURE 5 | Schematic representation of the actions of GH/IGF-1 in the ovarian granulosa cells. **(A)** In normal women, administration of GH in the early follicular phase exerts a synergistic effect with FSH on the production of estradiol. GH up-regulates the receptors for FSH (1) and increases the activation of aromatase by this hormone. The result is increased formation of estradiol from testosterone from the theca cells (2). **(B)** In rats, it has been shown that the up-regulation of FSH receptors induced by GH is mediated through activation of Signal Transducers and Activators of Transcription STATS (1). Ovarian GH increases the expression of IGF-1 and its receptor, and is IGF-1 which increases FSH-dependent aromatase activity (2). Bone morphogenetic proteins (BMP) suppress the expression of the ovarian GH/IGF-1 axis (3), therefore inhibiting the aromatization of testosterone to estradiol. In turn, GH/IGF-1 down-regulate the expression of BMP receptors (4). Blue arrows: stimulation. Red arrows, inhibition. **(C)** Systemic GH (1) and/or ovarian GH (2) increase progesterone production in ovarian granulosa cells induced by FSH (3), while BMP blocks this effect of GH (4). Blue arrows, stimulation; Red arrows, inhibition; BV, blood vessel; BM, basement membrane; FF, Follicular fluid.

the systemic IGF-1. As it has been described above GH facilitates implantation, most likely by increasing the production of matrix metalloproteinases and stimulating trophoblast cell proliferation which allows blastocyst cavity formation and invasion of the endometrium (72).

In summary, the GH/IGF-1 system (endocrine and autocrine or paracrine, or all of them) plays a very important function in practically all the physiological events occurring during a normal menstrual cycle, even facilitating the implantation and development of the embryo if pregnancy occurs. In this sense, we observed (unpublished data) that in six out of ten women in coma (aged between 28 and 39 years, Glasgow coma scale score 7–11, time in coma 2–10 years), produced by a traumatic brain injury or stroke, menstrual cycles reappeared and normalized (the patients were amenorrheic since their injury occurred), 10–18 months after beginning the administration of the hormone and rehabilitation in our Medical Center. This effect must be attributed to GH, since it is unlikely that the rehabilitation followed may have effects at the ovarian level or on the hypothalamic-gonadotropic axis, and there are no data indicating that this could be the case.

Growth Hormone and Menopause

The pituitary secretion of GH suffers strong changes throughout human life: very high release during the first year of life, a decrease during childhood followed by a new high release when puberty begins and then a progressive decrease in the amount of hormone released in each secretory pulse [for review, see (2)]. After age 20, more or less, GH secretion is progressively declining by one-half every 7–12 years (73), and it is accompanied by a decline in plasma levels of IGF-1, although its decrease is lesser than that occurring with GH (74). Therefore, at age 50, more or less, GH secretion is residual, if it exists, and it leads to significant changes in body composition, such as reduced muscle mass, increased adiposity, reduced energy, decline in sexual activity, and increased cardiovascular risk, among other symptoms (75). Given the beneficial effects of GH, the question is: why does GH secretion declines alongside aging? Deconvolution analysis of data obtained from blood sampling every 20–30 min in humans indicate that there are age-related alterations in the hypothalamic control of GH secretion, its modulation by gonadal steroids, and in GH autofeedback, leading to significantly decreased GH secretion in elder people (76, 77). This supports the pioneering

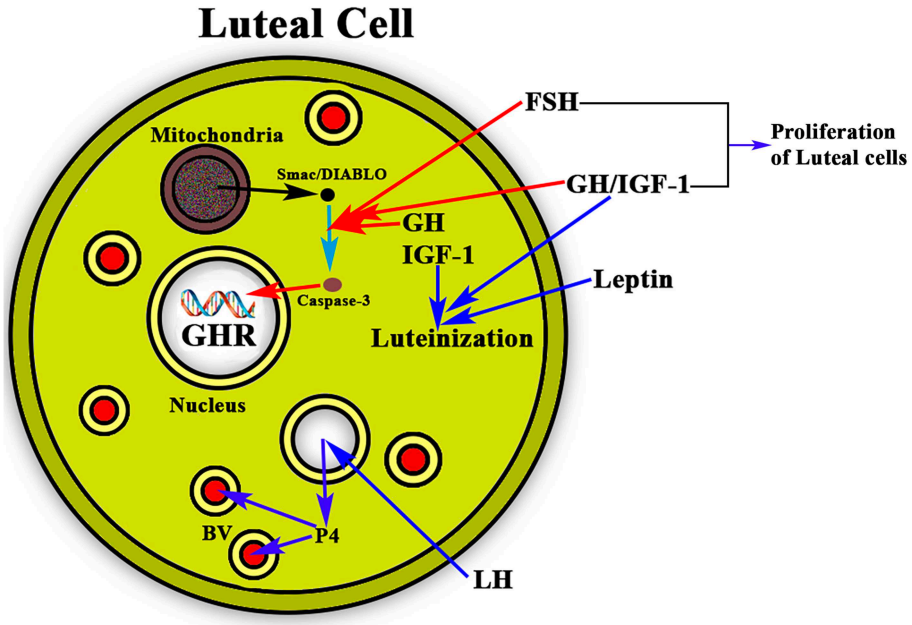

FIGURE 6 | Schematic representation of the actions of GH/IGF-1 in the ovarian corpus luteum. The figure shows a luteal cell in which the GHR can be see inside the nucleus. The production of progesterone (P4) is induced by LH, then P4 enters the bloodstream (BV; blue arrows) for playing very important functions in the body, mainly if conception exists, but also acting at many other levels, even in the brain. As shown in the figure, GH and IGF-1 are expressed in the luteal cells where they act synergistically with leptin for inducing the luteinization of the follicle that has ovulated (blue arrows). In addition, GH/IGF-1 together with FSH inhibit the possible apoptosis of the luteal cells (red arrows) by inhibiting the proapoptotic activity of caspase-3, which is stimulated by Smac/DIABLO released from the mitochondria. Moreover, GH/IGF-1 together with FSH induce the proliferation of luteal cells.

study conducted in 1985 in which it was demonstrated that the integrated concentration of GH during 24 h was strongly affected by the age of the individual (78) and also reinforces the role played by gonadal sex steroids in the neuroregulation of GH (**Figure 4A**).

Although GH secretion begins to decrease early in life, menstrual cycles are normally maintained until later ages (45–50 years). Menopause, the final interruption of menstrual cycling, occurs when the pool of ovarian follicles is fully depleted. This leads to a high increase in the production of Gns, with plasma FSH values that double or triple those of LH (**Figure 4A**). This reflects the loss of ovarian inhibit production, the main negative regulator of FSH secretion, and also the deficient production of estrogen by the menopausal ovaries, which leads to a greater release of LH. Consequently, although throughout this review, we provide a number of data showing the positive effects of GH in the ovaries and its role as co-gonadotropin, it seems that age-related GH deficiency is not the reason for the interruption of the normal ovarian function. In fact, the menopausal ovaries still release a small amount of E2, and the administration of 1 mg of this estrogen twice daily during 7–10 days to healthy post-menopausal women induces a two-fold amplified mass of GH secreted per burst, and an augmented amplitude and mesor of the 24-h rhythm in GH release (79). The effects of E2 on GH secretion after menopause were confirmed in a double-blind, controlled study in which plasma GH was analyzed before and after somatostatin infusion in healthy post-menopausal women treated with placebo or with an inhibitor of aromatase or a selective inhibitor of the E2 receptor. In this study a 1-h GH peak rebound after somatostatin infusion was clearly decreased during both estrogen-deprivation protocols (80). This study concluded that GH secretion after menopause depends on low levels of ovarian estrogens, and also confirms our previous postulate about that the interruption of somatostatin release induces the synthesis and secretion of GH via GHRH (9), and that sex steroids act on GH neuroregulation by inhibiting somatostatin secretion via alpha-2 noradrenergic pathways (9).

A recent study analyzed the effects of the administration of GH over 26 weeks on the nocturnal pulsatile secretion of LH and the levels of sex steroids in a group of healthy post-menopausal women. Their results indicate that GH did not exert any significant effect on the secretory dynamics of LH or plasma levels of LH, nor were plasma levels of sex steroids modified, although IGF-1 did increase significantly (81). This indicates that neither GH nor IGF-1 modulates the hypothalamic-pituitary-gonadal function in older post-menopausal women.

In all, these data indicate that although somatopause and menopause are more or less temporally related, once menopause begins the administration of GH seems to be totally ineffective at the ovarian level, likely related to the depletion of ovarian follicles while aging.

Growth Hormone and *in vitro* Fertilization in Poor Ovarian Responders

Based in the data presented in this review it is likely that GH administration may be useful as an adjuvant therapy in *in vitro* fertilization (IVF) for poor ovarian responders (POR) unable to get pregnant. In fact, the combined treatment with GH and gonadotropins was already used many years ago with successful results in terms of more follicles developed, more oocytes collected, and higher urinary estrogens in patients with polycystic ovaries (82, 83). Since those studies, several different trials have been conducted combining GH treatment with Gns or human chorionic gonadotropin to induce *in vitro* fertility and embryo transfer in POR, and although some contradictory results have been reported, the overall conclusion is that the addition of GH significantly improves pregnancies in these infertile women and the number of positive results in terms of live birth rate. In this line, a recent study in 62 older women showed that co-treatment with GH led to the preovulatory down-regulation of FSHR, BMPR1B, and increased density of the largest follicles, and improved fertility in these older women in which there was already a significant decrease in the ovarian follicular reserve (84). Another study analyzed the effects of 6-week pretreatment with GH in POR which were submitted to an *in vitro* fertilization treatment. This study, carried out in 380 POR, showed that the administration of the hormone significantly improved the rate of utilization of oocytes and embryo quality increasing the live birth rates, even in older patients who had previously experienced unsuccessful results from classical techniques (85). Moreover, another recent study demonstrated that co-treatment with GH in patients with normal ovarian response significantly increased pregnancy rate (86). Therefore, from these and other studies, it seems to be clear that GH plays a key role in ovarian fertility and Assisted Reproductive Techniques. An extensive and detailed analysis of these effects of GH as adjuvant therapy in IVF and embryo transfer can be seen in the review carried out by Li et al. (87).

OVARIAN ANGIOGENESIS

As is logical, and it has been briefly described before, for a normal ovarian functioning the gland needs to receive an adequate and perfectly regulated blood supply.

Follicles are the main functional structures of the ovaries; they are formed during fetal development and are composed of a single layer of cells, granulosa cells (GCs), that surround the oocyte. These are the primordial, inactive follicles; these GCs are surrounded by another type of cells, the thecal cells (TCs) which will play a very important role, producing sex steroids, in the development of the follicles until their final stage which culminates in ovulation and luteogenesis.

Before the beginning of the reproductive life, a number of primordial follicles have been transformed in primary follicles. These consist in the oocyte surrounded by the pellucid zone and GCs. Around these a basal membrane separates the GCs from the TCs. In each menstrual cycle, a small number of primary follicles grows until, in general, only one of them, the dominant follicle, suffer a proteolytic process that allows the release of the ovule, while the other follicles that had initially begun to evolve together with the dominant follicle, progressively suffer a process of atresia. The question is: why does only one follicle ends the process leading to ovulation in each menstrual cycle?

For years it was believed that this was due to the different aromatase activity that transformed the testosterone produced in the TCs into estradiol (E2) in GCs (**Figure 2**); the greater the amount of this steroid, the greater the follicular growth capacity, a characteristic of the dominant follicle. Most likely this concept is valid, but: what determines the amount of aromatase in each follicle? Years ago, it was demonstrated that in the adult ovary the vasculature is not distributed uniformly among the ovarian follicles (10). Primordial follicles and slow-growing preantral follicles only have blood supply from vessels existing in the surrounding stroma. This implies that an adequate vascular supply is a rate-limiting step in the selection and maturation of a dominant follicle (88), while those follicles with insufficient blood supply would have limited their growth and would suffer atresia; in fact, the existence of a correlation between increasing levels of the vascular endothelial growth factor A (VEGF-A) and E2 in follicular fluid agrees with the idea that follicles with highest levels of VEGF-A will grow until reaching a preovulatory state (89) (**Figure 7**). As the follicular development and sex steroids production is under the control of pituitary Gns and some other growth factors, both concepts are compatible. Moreover, this reinforces the important role of angiogenesis in the ovarian function. In fact, blocking VEGF-A effects on the ovary leads to marked decrease of proliferation in the theca of secondary and tertiary follicles and also decreases GCs proliferation and the subsequent production of sex steroids.

How Ovarian Angiogenesis Is Regulated?

Fibroblast growth factor-2 (FGF-2) was the first factor identified as pro-angiogenic in the ovary (90, 91), being expressed in the mature follicle and corpus luteum. However, given its effects on many different cell types, it is not considered as a factor of interest in the ovarian angiogenesis. In fact, its ovarian expression shows small variations during a menstrual cycle in primates and other mammals. The same occurs with many other pro-angiogenic factors which are not specific of vascular endothelial cells (3).

As stated above, follicular development implies the proliferation and differentiation of GCs; the same occurs with the surrounding TCs. These processes are induced by pituitary Gns, and continue until the dominant follicle, after a sudden and high luteinizing hormone (LH) surge, releases the mature ovule and CL is formed and maturates producing mainly progesterone (P_4). If there is no conception, this CL undergoes important morphological and functional changes that lead to its regression and formation of a residual structure, as is the corpus albicans (90), in which degeneration of the vasculature exists (88). This succession of events in a menstrual cycle occurs in parallel with rapid and continuous changes in the irrigation and functionality of the ovarian structures involved (92). In fact, in the CL there is a high density of capillaries (**Figure 7**), as they are microvascular endothelial cells the most abundant cell type in this structure; even more so, each luteal cell is in contact with

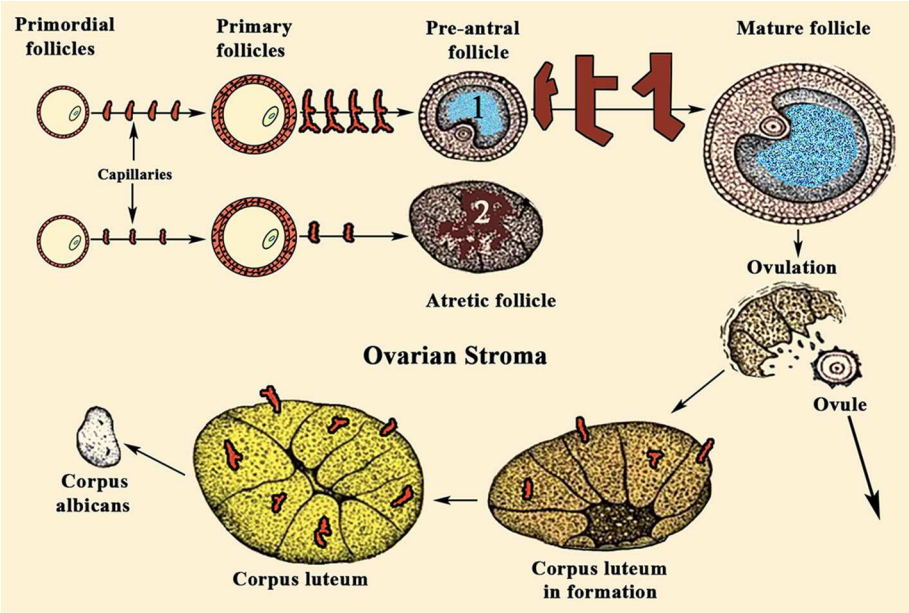

FIGURE 7 | Sequence of ovarian events during a normal menstrual cycle. During a normal menstrual cycle some primordial follicles begin to grow but, usually, only one of them reach the final maturation until it releases the ovule. It depends on the amount of VEGF-A and E2 existing in the follicular fluid. (1) Shows a dominant follicle, in which VEGF-A and E2 levels are quite higher than in the follicles that began to grow with it. These follicles degenerate and become atretic (2). Note the high number of capillaries supplying blood to follicle (1), while the atretic follicle (2) is almost privated of blood supply. VEGF-A, among other factors, is the main responsible for the generation of new small vessels supplying blood to the follicles. Similarly, after ovulation, the formation of the corpus luteum requires an increase in blood supply, as can be seen in the figure.

one capillary (92, 93), most likely to increase the production of P_4, key for the beginning of pregnancy.

The first factor identified as main responsible of the vascular changes occurring during a menstrual cycle was VEGF-A (94). In the pre-ovulatory follicle, the granulosa layer is avascular, while the theca is strongly vascularized. During follicular development VEGF-A is accumulated, until LH induces proteolytic activity and the basement membrane is degraded; this leads to the release of VEGF-A which induces migration of endothelial cells to GCs, endothelial proliferation and sprouting of pre-existing vasculature, tube formation and recruitment of pericytes, as well as, perhaps in conjunction with other angiogenic factors, vessel stabilization, and maturation (95). These actions of VEGF-A are mediated by the VEGF-A receptor 2 (VEGF-AR2), while VEGF-AR1 seems to play a negative role suppressing signaling through VEGF-AR2.

Another group of endothelial-specific factors detected in the ovary are Angiopoietins (Ang) (96, 97), of which Ang2 seems to act, in the absence of VEGF-A, as an inducer of ovarian vessels destruction. This effect is produced by the binding of Ang2 to the Ang1 receptor Tie2, therefore impeding the binding of Ang1. Thus, Ang2 acts in an opposite manner than the isoform Ang1 who, like VEGF-A, is essential for normal vasculature development (97), and stabilization of newly formed blood vessels. However, in the presence of VEGF-A increased autocrine expression of Ang2 by the vascular endothelium induces angiogenesis. Perhaps these divergences explain the reason by which Ang1 and Ang2 are differentially expressed in the ovary during a normal menstrual cycle. Interestingly, pituitary gonadotropins, in particuar LH, have been demonstrated to be major inducers of angiogenesis and VEGF-A/Ang expression in the ovary. The midcycle surge of LH strongly stimulates VEGF-A and Ang expression in GCs in many species, including primates (98) (**Figure 8**), while the administration during 3 days of a Gn-releasing hormone (GnRH) antagonist, which leads to the blockade of LH secretion, clearly decreased VEGF-A expression in the CL of primate ovaries (99). However, this effect of LH on ovarian VEGF-A expression may be modulated or be dependent of local ovarian factors, such as ovarian sex steroids (100), as occurs in other tissues. This agrees with the concept that the VEGF-A ovarian expression changes cyclically throughout a menstrual cycle (101).

Pituitary Gonadotropins and Ovarian Angiogenesis

The effects of LH on the ovarian production of VEGF-A have been confirmed in infertile, but otherwise normal, women to which recombinant LH (rLH) was administered (75 U/day) during the late follicular phase, induced by administration of rFSH, in which the plasma and follicular liquid levels of VEGF-A, and its soluble receptor sFlt-1, were evaluated. The results obtained in this pioneer study indicated that rLH significantly increased the ratio VEGF-A/sFlt-1 in the follicular fluid, indicating that LH induced ovarian follicular angiogenesis (92), while inhibiting VEGF-A by treatment with truncated

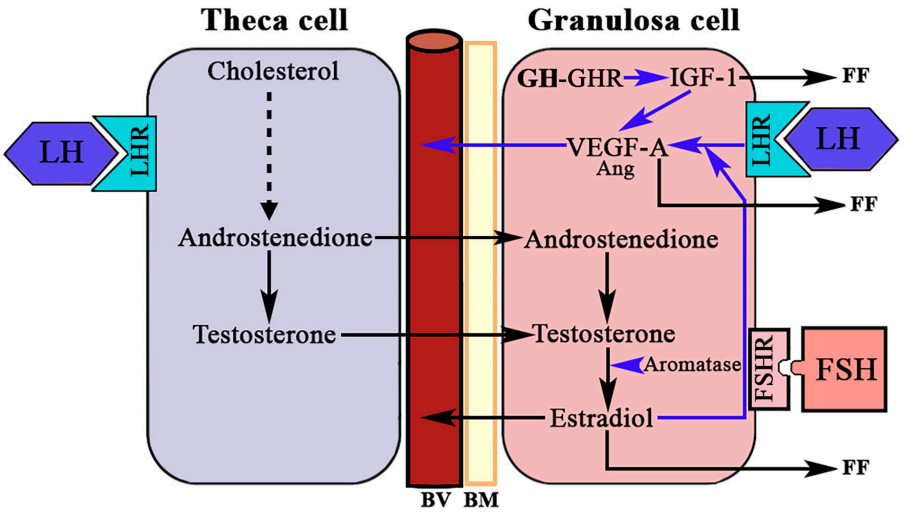

FIGURE 8 | LH, GH/IGF-1, and ovarian angiogenesis. The androgens produced in the theca cell under the stimulation of LH cross to the granulosa cell where, under the stimulation of FSH, they are aromatized to estradiol. In the growing follicle, there are receptors for LH that in combination with the produced E2 induces the expression of VEGF-A (mainly) and angiopoietin 1 (Ang). VEGF-A and Ang 1 induce the formation of new vessels (BV). There is a possibility that the GH produced in the granulosa cells induces the expression of IGF-1 who also favors the production of VEGF-A. VEGF-A and estradiol are released into small blood vessels, and both factors and IGF-1 are also released into the follicular fluid (FF). BV, blood vessels; BM, basement membrane; LHR, LH receptor; FSHR, FSH receptor; GHR, GH receptor; Blue arrows, stimulation.

Flt-1Fc (vascular endothelial growth factor trapA40) leads to the inhibition of angiogenesis during follicular development in primates (102).

Although the effects of LH on ovarian angiogenesis are clear, some data indicate that FSH may also play a role in this process. For instance, it has been demonstrated that the addition of 0.3 IU/mL of FSH during ovarian cryopreservation by vitrification increased the revascularization and follicular survival for mouse ovarian grafts up-regulating angiogenesis and ovarian survival factors (103); this may preserve ovarian fertility avoiding the follicular damage produced by chemotherapy and/or radiotherapy. Recently, it has been demonstrated that interleukin-6 (IL-6), a major factor in the regulation of VEGF-A expression (104), also promotes the expression of VEGF-A induced by FSH in bovine GCs (105), suggesting that there is a synergistic relationship between FSH and IL-6 in the regulation of VEGF-A expression. This effect of IL-6 on VEGF-A is dependent on the promotion by IL-6 on the VEGF-A regulators HIF-1α (hypoxia inducible factor-1α) and COX2 (cyclooxygenase2), since their inhibition significantly decreases VEGF-A expression in GCs. In any case, it seems that a direct role of FSH in ovarian angiogenesis is restricted to particular non-physiological situations.

Growth Hormone and Ovarian Angiogenesis

Among the factors regulating VEGF family expression in humans GH plays a pivotal role, either directly or by inducing the expression of other proangiogenic factors, such as the Insulin-like growth factors (IGF-1 and IGF-2), FGF-2, epidermal growth factor (EGF), among others; moreover, GH is able to interact with receptors for Prolactin (PRL), which also is able to induce proangiogenic effects [for review, see (106)]. At this point, it is important to note that the pituitary secretion of GH is strongly potentiated by sex steroids, mainly E2, which in its free form (fE2) directly reaches the central nervous system (CNS), or is formed at this level from the hypothalamic aromatization of testosterone (9).

The possibility exists that systemic GH could induce ovarian VEGF-A expression, but this has not been demonstrated; perhaps because the own GH and its receptor are produced in the ovary in humans and bovines (11, 13, 107). Moreover, the production of GH by ovaries is higher in GCs and oocytes, avascular follicular compartments, separated from the systemic circulation by the basal lamina (8) (**Figure 4**). Local expressions of GH and GHR have also been detected in the chicken ovary during sexual maturation in hens (108, 109) and fishes (110). Ovarian GH would act in an autocrine/paracrine way, so the hormone could play a role in the regulation of ovarian angiogenesis, as suggested by the fact that ovarian expression of the GH gene increases during follicular development (11), but significantly decreases when immature follicles reinitiated meiosis (111). The cyclic expression of follicular GH shows parallelism with the expression of follicular GHR in some species analyzed, including humans (13, 108, 112). If this temporary pattern of ovarian GH-GHR correlates with changes in ovarian VEGF-A during a normal menstrual cycle, has not yet been established. However, the fact that the expression of VEGF-A in the follicle is related to the size of the follicle itself (**Figure 7**) is crucial to understand why angiogenesis is important and why GH, the main hormone involved in tissue growth, should play an important role in the production of VEGF-A in the ovary (89).

How the ovarian synthesis of GH is regulated is not known, although, as described above, GH-releasing hormone (GHRH) mRNA and GHRH receptor were found many years ago in humans and rats ovaries (112, 113). However, differently to that occurring at the hypothalamic-pituitary axis, ovarian GHRH does not seem to stimulate the synthesis and secretion of ovarian GH. In fact, recently it has been reported that a GHRH homodimer up-regulates the ovarian GHRH receptor increasing the development and maturation of follicles without affecting ovarian GH production, at least in an animal model (114). Another important GH secretagogue, such as ghrelin, has been found to increase GH secretion, but not GH synthesis, in porcine follicles *in vitro* (115), and ghrelin and its receptor have been found in the ovaries of pig and hen (116, 117). However, administration of ghrelin does not induce ovarian GH release in cultured ovaries in chicken (118). Paradoxically, ovarian GH stimulates ghrelin synthesis and secretion (115).

It cannot be ruled out that GH can act on the ovarian angiogenesis through one of the multiple mediators of the actions of the hormone, mainly IGF-1 (119). There is an ovarian production of IGF-1 and its receptor that induces angiogenesis by stimulating the production of VEGF-A in luteal cells, and also by stimulating the proliferation and differentiation of endothelial cells (120, 121). IGF-1 can act directly or as a mediator of the proangiogenic effects of GH (119). GH stimulates IGF-1 expression in rat and porcine granulosa cells (61, 122), and IGF-1 antibodies block the effect of GH on oocytes maturation in rat follicles (123). However, a study indicated that GH administration does not induce the synthesis of IGF-1 in human pre-menopausal ovaries (124), although previous studies showed that GH increases IGF-1 in follicular fluid in a number of species, including humans (**Figure 5**) [for review, see (14)]. Therefore, the GH effects on ovarian IGF-1 production may be exerted via endocrine or auto/paracrine actions.

Other proangiogenic growth factors, present in the ovary and whose transcription is induced by GH, such as FGF-2 and EGF, among others, seem not to play any key role on ovarian

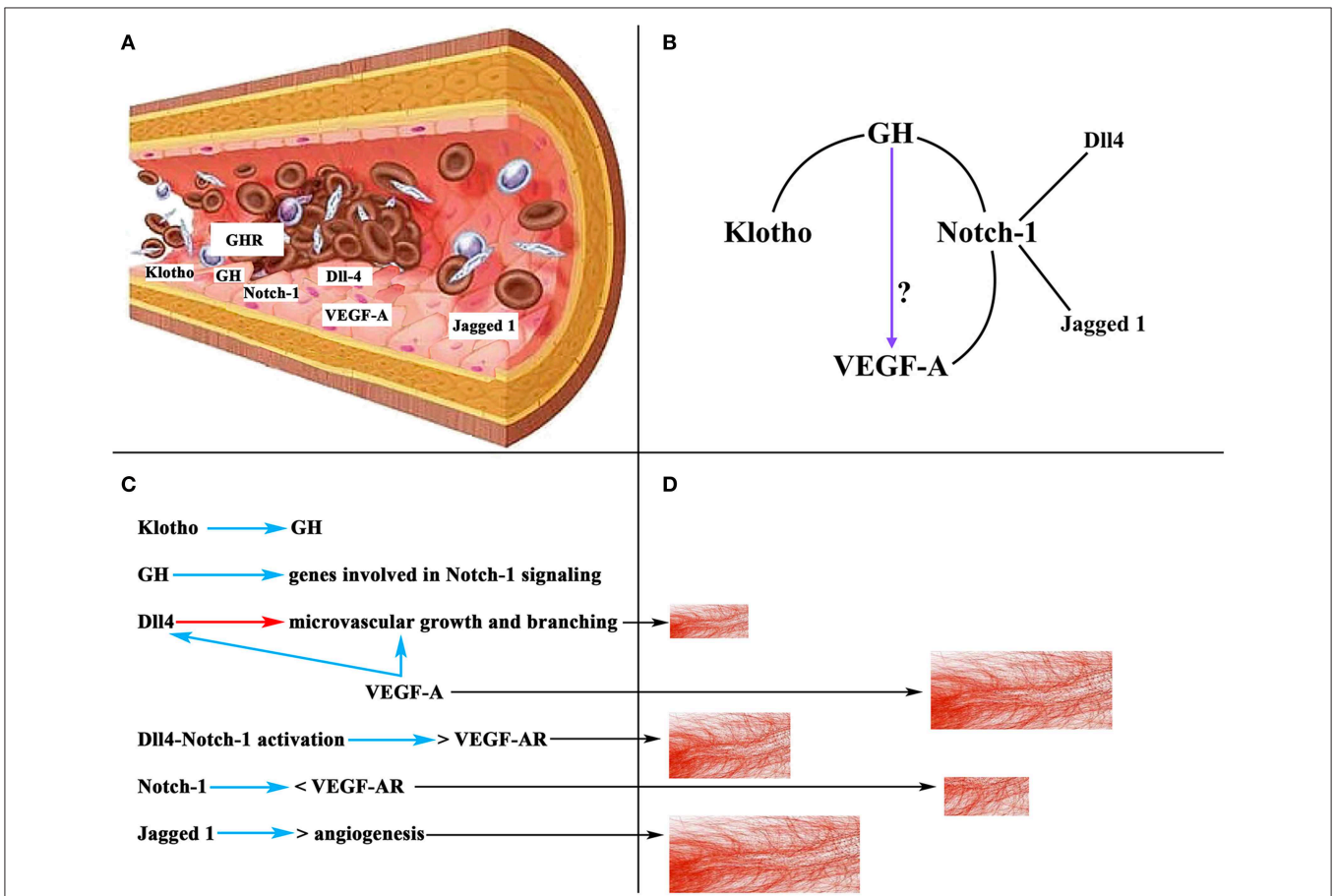

FIGURE 9 | Schematic representation of the action of VEGF-A, Notch-1 and its ligands on ovarian angiogenesis. **(A)** Section of an ovarian vascular vessel showing the expression of some factors involved in ovarian angiogenesis: Klotho, VEGF-A, Notch-1 and its ligands Dll4 and Jagged 1, the receptor for GH (GHR), and presumably GH. **(B)** Relationships between Klotho, GH, Notch-1, and VEGF-A. These lead to the possibility that GH can act directly on the induction of the expression of VEGF-A in the endothelium of ovarian microvessels. **(C)** Summary of the actions of the factors mentioned in **(A)**. Blue arrows, stimulation; Red arrow, inhibition; <, decrease; >, increase; VEGF-AR, receptor for VEGF. **(D)** Schematic representation of the growth of ovarian microvessels produced by the action of each one of the factors showed in **(C)** (black arrows).

angiogenesis, although they can contribute to it; rather they participate in steroidogenesis, follicular development, and luteal function (125).

Of interest here is the relationship between GH, Notch-1, and VEGF-A. GH has been shown to regulate the ovarian expression of some genes involved in Notch-1 signaling to induce repair and regeneration of ovaries in mice with premature ovarian failure, as well as to induce E2 secretion and oocyte maturation (126). We will not analyze here the important effects that Notch-1 plays in the organism, but only the most important ones on angiogenesis in the ovary (127). In mice, during the follicular phase Notch-1 is expressed in the endothelium of the thecal layer, while in the luteal phase Notch-1 has been detected in endothelial cells from new vessels of the CL and mature vessels of the thecal layer. In primates, two important Notch-1 ligands, such as delta-like protein Dll4 and Jagged 1 (126, 128), regulate angiogenesis directly in the endothelium, being specifically expressed at sites where this angiogenesis occurs (129). Dll4 regulates microvascular growth and branching induced by VEGF-A to prevent excessive branching that could lead to vascular dysfunction (130), whereas the blockade of Dll4 with an anti-Dll4 monoclonal antibody leads to an increase in luteal angiogenesis and a greater density of microvessels in the primate ovary (131). In turn, inhibiting Jagged 1 induces anti-angiogenic effects (132). Based on these data it is evident that Notch-1 plays an important role in ovarian angiogenesis. Curiously, there is a strong relationship between VEGF-A and Notch signaling. VEGF-A increases Dll4 expression in endothelial cells *in vitro* (133), and Dll4-Notch-1 activation induces an increase in the expression of VEGF-A receptors in cultured endothelial cells (134), although activation of Notch-1 alone induces a reduction in the expression of VEGF-A receptor 2 (134). All this may be related to the need to form and maintain an adequate number and functionality of the ovarian blood vessels for a normal ovarian function in each of the phases of a menstrual cycle. In fact, ovarian angiogenesis is abnormal in women with polycystic ovarian syndrome; in these patients, there is an increase in follicular vascularization and vascular permeability (135). The excessive ovarian vascularization is responsible for the pathologic characteristics of the syndrome (127). **Figure 9** shows a schematic representation of the effects of VEGF-A, Notch-1 and its ligands on ovarian angiogenesis.

As stated above, there is no evidence to show that GH induces the ovarian expression of VEGF-A; however, the possibility exists that ovarian vessels produce GH and this hormone promotes the expression of VEGF-A. In fact, the vascular endothelium is an important gland of internal secretion that produces a series of growth factors, including GH (136) (**Figure 9A**), that act in a paracrine/autocrine fashion. In addition, there is a specific GHR in the vascular endothelium (137, 138) (**Figure 9A**), and the damaged vascular endothelium produces Klotho, which stimulates the pituitary secretion of GH, but perhaps also the production of endothelial GH to repair the vascular endothelium when it is damaged (3). Therefore, given the explained relationships between Notch-1 and VEGF-A, and Notch-1 and GH, and Klotho and GH, it seems feasible that GH may directly participate in the regulation of ovarian angiogenesis (**Figure 9B**).

In summary, it is clear that adequate angiogenesis is critical in each of the phases that a primary follicle has to follow until it is transformed in an ovulatory follicle, ovulation occurs, and CL is formed and maintained until pregnancy takes place. Abnormal angiogenesis not only impedes the physiological evolution of this process but also may be the cause of pathologies such as infertility, polycystic ovarian syndrome and even ovarian cancer. Ovarian angiogenesis is very complex, and although VEGF-A plays a key role in it there are many factors, still little known, that can influence the actions of this protein both positively and negatively.

CONCLUSIONS

In conclusion, it is clear that GH, expressed in the ovary and/or systemic GH, is very important in all stages of ovarian development and normal functioning until menopause. The ovarian effects of GH can be exerted by the hormone itself and/or through its mediators. Of interest is the bidirectional inverse relationship between the actions of GH and BMP at the ovarian level, as well as their actions on the secretion of gonadotropins. It is likely that GH can be a very important factor as adjuvant therapy for IVF and embryo transfer in infertile women poor ovarian responders. In addition, ovarian angiogenesis plays a key role in the normal ovarian functioning since puberty begins until menopause. The formation and functionality of the ovarian vessels depend mainly on VEGF-A, although Angiopoietins also play a role in ovarian angiogenesis. Ovarian expression of VEGF-A is regulated by LH together with sex steroids, but GH also appears to be (directly or indirectly) actively involved in this process.

AUTHOR CONTRIBUTIONS

JD conceived, structured, and wrote the text. DC contributed to writing the section devoted to Ovarian Angiogenesis.

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Application of Growth Hormone in *in vitro* Fertilization

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Growth hormone (GH) is a peptide hormone secreted mainly by the anterior part of the pituitary gland and plays a critical role in cell growth, development, and metabolism throughout the body. GH can not only directly influence human oocytes and cumulus cells but also indirectly improve oocyte quality through activating synthesis of insulin-like growth factor-I or promoting follicle-stimulating hormone-induced ovarian steroidogenesis. Since GH can regulate female and male infertility, it has been applied in the management of infertility for many years, especially in patients with poor ovarian response or poor prognosis. During ovarian stimulation, GH administration might improve the success rate of *in vitro* fertilization (IVF) probably through the beneficial effects of GH on oocyte quality as indicated by a higher number of mature oocytes and embryos arriving at the transfer stage and a higher fertility rate in GH-treated patients. However, there is still great controversy in the application of GH in IVF. While some researchers showed that pregnancy, implantation and live birth rates could be increased by ovarian pretreatment with GH, others did not support GH as an effective adjuvant for infertility treatment because the live birth rate was not increased. This study reviewed and summarized recent advancements and benefits in clinical application of GH, trying to reach a just unbiased conclusion regarding the effect of GH therapy in IVF.

Keywords: growth hormone, *in vitro* fertilization, infertility, reproduction, effect

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INTRODUCTION

As a peptide hormone secreted mainly by the anterior part of the pituitary gland in a pulsatile manner, growth hormone (GH) plays a critical role in cell growth, development and metabolism throughout the body with multifunctional effects ranging far beyond the effect on linear growth (1). Human oocytes and cumulus cells have GH receptors (GHRs) and can be directly influenced by GH, and GH can promote nuclear maturation of denuded human oocytes (2–4). GH may also have an indirect effect on improving oocyte quality through activating synthesis of insulin-like growth factor (IGF)-I or promoting follicle-stimulating-hormone-induced ovarian steroidogenesis (5, 6). Since GH is involved in the regulation of female and male infertility, it has consequently been applied in the management of infertility for many years (7), especially in patients with poor ovarian response or poor prognosis (8–11). During ovarian stimulation, GH administration can improve the success rate of *in vitro* fertilization (IVF) (12, 13) probably through the beneficial effects of GH on oocyte quality as indicated by a higher number of mature oocytes and embryos arriving at the transfer stage and a higher fertility rate in GH-treated patients (8, 10, 11, 14). Pregnancy, implantation and live birth rates can be increased by ovarian pretreatment with GH in many studies (5, 9, 12, 13, 15–17), and subgroup analysis in a systematic review and meta-analysis indicated

that GH administration with gonadotropin significantly increased the clinical pregnancy (risk ratio (RR) 1.76, 1.25–2.48), live birth rate (RR 1.91, 1.29–2.83), collected oocytes number (standard mean difference (SMD) 1.09, 95% CI 0.54–1.64), MII oocytes number (SMD 1.48, 95% CI 0.84–2.13) and E2 on human chorionic gonadotropin day (SMD 1.03, 95% CI 0.18–1.89) among patients with poor ovarian responses (11). However, great controversy still exists in the application of GH in IVF, with some randomized controlled trials presenting no definitive benefits in increasing the live birth rate for poor responders (18, 19). Dakhly et al. reported non-significant ($P > 0.05$) improvement in the live birth (17.5 vs. 14.1%) and cumulative live birth rate (18.3 vs. 14.7%) in a randomized controlled trial ($n = 120$ patients for each group) with addition of GH as an adjuvant therapy [2.5 mg or 7.5 IU GH injected subcutaneously from day 21 of the previous cycle along with GnRHa until the day of human chorionic gonadotropin (hCG)] to the long down regulation protocol in poor responders undergoing IVF, even though GH significantly ($P < 0.001$) increased the endometrial thickness (11.8 ± 1.3 vs. 11.3 ± 1.2 mm), number of collected oocytes (5.4 ± 1.7 vs. 4.3 ± 2.1), MII oocytes (4.1 ± 2.1 vs. 2.1 ± 1.4), fertilized oocytes (4.0 ± 2.2 vs. 2.0 ± 1.2), transferred embryos (2.4 ± 0.9 vs. 1.6 ± 1.1) and frozen embryos (1.1 ± 1.4 vs. 0.2 ± 0.5) (18). However, their outcomes were questioned by Yovich et al. (20) who believed that this randomized controlled trial suffered from some major limitations (including suboptimal dosage of Cyclogest 400 mg twice daily in the luteal phase management and use of a non-single embryo transfer strategy) which strongly weakened the effects. Dakhly et al. also acknowledged that further studies were needed to investigate the true impact of adding GH to the induction protocols in poor responders (20). In the most recent randomized placebo controlled trial investigating the effect of adding human GH to an IVF cycle in previously documented poor responders to FSH with GH administered at the dose of 12 IU starting on day 1 of stimulation and stopped on the evening of hCG scheduling for egg pickup (19), a significant increase in the live birth rate (14.5% for GH vs. 13.7% for placebo, with an odds ratio (OR) 1.07 and 95% CI between 0.37 and 3.10) from the addition of GH could not be found. However, this clinical trial suffers from too many limitations and has to be interpreted with caution. To achieve the goal of increasing the live birth rate from 10 to 20% by addition of GH according to previous systematic reviews and ability to recruit, 195 participants were needed for each arm in order to reach the 5% significant level, 80% statistical power and 10% dropout or cancellation rate, however, only 65 patients were recruited and assigned to either the GH group or the controlled placebo group from four clinical centers. This indicated that the trial was not a successfully completed trial without sufficient statistical power, implying that their outcomes were underpowered. This clinical trial lasted 4 years and was ended early as the provided drug had expired, indicating that this trial was terminated because of the expired drug rather than insufficient effect of GH administration on these poor-responding patients. Usually, when a clinical trial is terminated ahead of time, it is because the targeted endpoint has been proven in advance or because of substantial side effects of the target drug. However, this trial was terminated in advance because of the expired drug without even achieving the planned endpoints or goal of study. Moreover, as stated by the authors, some women who had good responses were also enrolled because different definition of poor responders was adopted (their definition does not fit with all classical international criteria) in this trial, to say nothing of many other limitations listed at the end of the study (19). Since different opinions exist in the application and benefit of GH adjuvant therapy in IVF, the present review aims to review and summarize recent advancements and benefits in clinical application of GH, trying to reach a just unbiased conclusion regarding the effect of GH adjuvant therapy in IVF based on the following aspects: theoretic bases of GH application, GH application in IVF, administration protocol and subjects and benefits of GH application (**Table 1**).

TABLE 1 | Effects and application of growth hormone in *in vitro* fertilization.

Theoretic bases of GH application	GH application in IVF	Administration protocol	Subjects and benefits of GH
Signaling pathway	Improving ovarian response	4–6 w before hCG administration	Subjects: poor or normal ovarian responders
On ovarian reactivity	Improving oocyte quality	Luteal phase of preceding menstrual cycle	Subjects: poor quality of embryos
On follicle development	Improving uterine receptivity	Start of gonadotropin	Subjects: improper endometrial reaction
On endometrial receptivity		Middle and late follicular phases	Subjects: repeated implantation failure Benefit: increased live birth rate

GH, growth hormone; IVF, in vitro fertilization; hCG, human chorionic gonadotropin.

THEORETICAL BASES FOR GH APPLICATION IN IVF

GH is a key factor for optimal fertility in women, which has been proved by declined fertility in GH deficiency women and capability of GH replacement to capacitate successful unassisted pregnancies in previously infertile women with GH deficiency (21, 22). GH is produced and secreted not only by the pituitary but also locally by the gonads, uterus, placenta and mammary glands (7, 23). Different from the sexually dimorphic pulsatile nature of the pituitary secretion of GH, the GH secreted outside the pituitary is produced more continuously at lower concentrations (24, 25). GHRs are expressed in ovarian granulosa, theca cells, oocytes, cumulus cells, mammary glands, placenta and uterus (23). Binding of GH with its receptors can activate the JAK-STAT (Janus kinase-signal transducer and activator of transcription) pathway (26) to adjust steroidogenesis and gametogenesis, promote proliferation, development and maturation of the gonadal cells and follicles, and regulate secretion and response of gonadotropins besides improving endometrial receptivity and embryos quality.

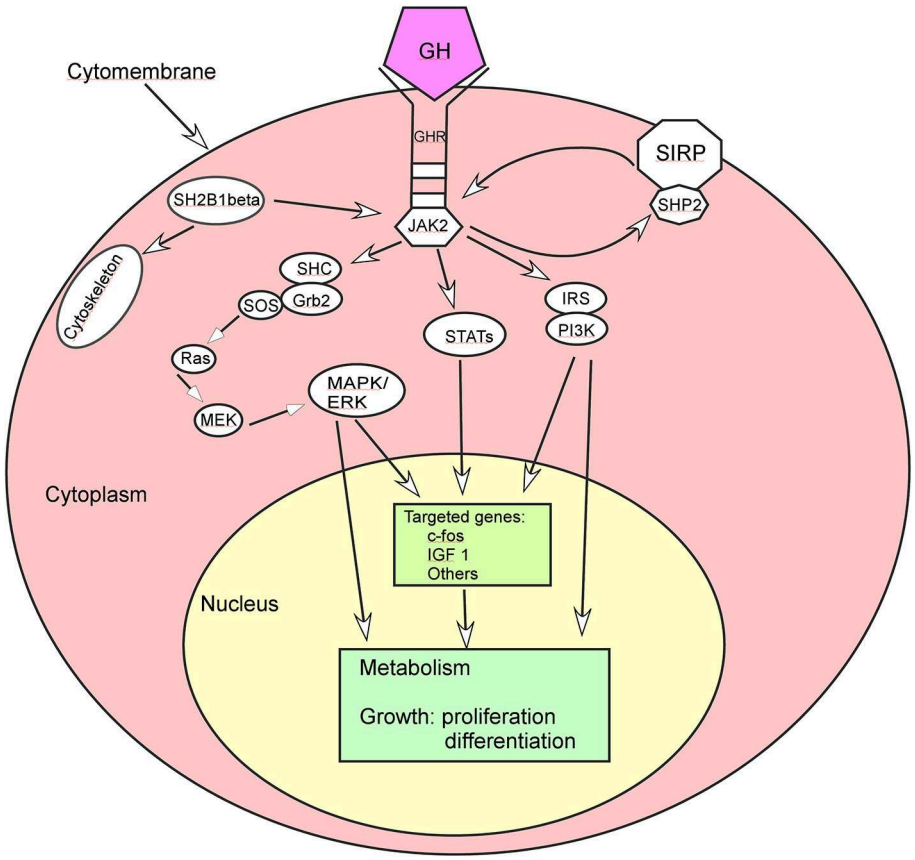

FIGURE 1 | Growth hormone (GH) acts through some signal pathways. ERK, extracellular signal-regulated kinase; GHR, growth hormone receptor; Grb, growth factor receptor-bound protein; IRS, insulin receptor substrate; IGF 1, insulin-like growth factor 1; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinase; MEK, dual specificity mitogen-activated protein kinase 2; PI3K, phosphatidyl inositol 3 kinase; SHC, SH2-domain containing transforming protein; SIRP, signal regulatory protein; SOS, son of sevenless; STAT, signal transducer and activator of transcription.

Signaling Pathway of GH Action

GH exerts its diverse effects on body growth and metabolism by binding to its membrane-bound receptors (**Figure 1**). The binding of GH to its receptors increases binding of JAK2 to the GHR, activates JAK2 and stimulates tyrosyl phosphorylation of both JAK2 and GHR (27). Activation of JAK2 is a critical initial step and activates multiple signaling pathways and cellular responses for GH effects, including 1) STAT transcription factors in the expression of multiple genes like the gene encrypting IGF-1, 2) SHC adaper proteins which activate the Grb2-SOS-Ras-Raf-MEK-ERK 1,2 pathway, 3) insulin receptor substrate (IRS) proteins in the phosphatidylinositol-3-kinase (PI3K) and Akt pathway, 4) signal regulatory protein α (SIRPα), a transmembrane scaffold protein which recruits proteins like the tyrosine phosphatase SHP2, and 5) SH2B1 which is a scaffold protein activating JAK2 and enhancing GH regulation of the actin cytoskeleton.

In the STAT transcription factors implicated in the expression of multiple genes, GH binding to GHR activates JAK2 which in turn phosphorylates GHR on multiple tyrosines to subsequently recruit various STAT proteins. These STAT proteins are phosphorylated in turn by JAK2 on a critical tyrosine. After phosphorylation, the STAT proteins are released from the GHR/JAK2 complex before dimerization and move to the nucleus to bind to the STAT binding sites in GH-regulated genes, affecting metabolism, growth and development of cells.

GH binding to its receptor can also activate the MAPK (mitogen-activated protein kinase) pathway by promoting binding of the Src homology 2 (SH2) domain of SHC adapter protein to JAK2-GHR complexes, tyrosyl phosphorylation of the three forms of SHC, and binding of the adapter protein Grb2 to SHC, regulating the target genes and subsequent cell growth and differentiation (28, 29). GH can promote association of the guanine nucleotide exchange factor SOS with SHC and activate Ras, Raf, MEK and Erks (extracellular signal-regulated kinase) via a SHC-Grb2-SOS-Ras-Raf-MEK-Erk1/2 pathway. Erks adjust some different kinds of molecules like protein kinases, phospholipases, cytoskeletal proteins, and transcription factors, thus exerting multiple effects in GH targeted cells (30).

Another pathway that GH regulates is the IRS-PI3K (insulin receptor substrate-phosphatidylinositol-3-kinase) pathway: GH stimulates tyrosyll phosphorylation of both IRS 1 and 2, binding

of the p85 regulatory subunit of PI3K to IRS 1 and 2 and tyrosine phosphatase SHP 2 to IRS 2, thus regulating glucose transport and other cellular responses (31–33). Activation of the IRS proteins by GH also indicates a pathway through which GH activates the transcription factor C/EBPβ. Activation of PI3K can convert phosphatidylinositol (3,4)-bisphosphate (PIP2) lipids to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to recruit Akt to the plasma membrane for the kinase PDK1 to access and phosphorylate T308 in Akt, resulting in partial activation of Akt (34). Akt phosphorylation of glycogen synthase kinase 3 (GSK3) leads to inhibition of GSK3 activity which decreases phosphorylation of GSK3 phosphorylation site in C/EBPβ, and elevated binding of a form of C/EBP designates liver activating protein (LAP) to the c-fos promoter for regulating cell proliferation and differentiation (35). The maximal expression of c-fos needs input from multiple GH signaling pathways, and the promoter region of c-fos includes a binding site for STAT 1 and 3 hetero or homodimers whose binding enhances c-fos gene expression (36–38).

SIRPα is a transmembrane glycoprotein recruits multiple SHP2 proteins, and activation of JAK2 by GH binding to GHR can highly phosphorylates SIRPα1 and recruits SHP 2 tyrosine phosphatases for negative regulation of GH-JAK2 signaling. SH2B1 is a scaffold protein to activate JAK2 and enhance GH regulation of the actin cytoskeleton.

Effect of GH on Ovarian Reactivity

As one of the targets of GH action, the ovary can be directly regulated by GH for its reactivity to gonadotropins. At the same time, GH can indirectly influence the ovarian function through IGF-I. Ovarian granulosa cells produce IGFs and express IGF receptors, and the IGFs and the receptors form a paracrine/autocrine system together with IGF binding proteins (6). Binding of IGF-I with its receptor can activate the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway to stimulate and regulate normal follicular growth and development in synergy with gonadotropins to increase the luteinizing hormone receptor level, consequently raising the ovary sensitivity to the follicule-stimulating hormone (FSH) (5).

Effect of GH on Follicle Development

GH is a potent activator of proliferation and differentiation of the ovarian follicles, and its administration generally increases ovarian weight and follicular size and number but inhibits follicular atresia (6, 39). GH is necessary for optimal follicular maturation and survival because GH addition to *in vitro* maturation medium of primordial and immature follicles can promote activation, survival and development of preantral follicles originating from sheep, goats and mice (40–42). Besides enhancing proliferation of the thecal and granulosa cells in the immature preantral follicles of mice (43), GH can improve the oocyte retrieval and fertilization rate in human oocytes subjected to *in vitro* maturation (44) and promote cumulus expansion and subsequent embryo development in rhesus macaque (45). *In vivo* GH can also improve the number of developing follicles in mice, buffalo and sheep (46–48), promote proliferation and inhibit apoptosis in the ovarian stroma and small follicles in chickens (49) as well as increase the follicle size in undernourished cows (50). Expression of GH in transgenic mice and adult sheep can promote follicular development besides increasing the ovary weight, ovulation rate, and the size and health of ovarian follicles (51, 52). In goat antral follicles, GH can indirectly adjust the early development stage but control the late stage formation of follicles through the GHR (53), and supplementation of GH to *in vitro* culture of caprine preantral ovarian follicles can increase the antrum formation rate, percentage of oocytes resuming meiosis and mature oocytes (42). This is because GH can induce granulosa and thecal cells to produce IGF-I, which can regulate the ovarian function to resume meiosis of the oocytes through autocrine/paracrine function. GH can also improve the mitochondria activity to directly ameliorate the oocyte quality (54). With aging, the number of functional mitochondria will be decreased, leading to impaired separation of chromosome associated with failed fertilization. Administration of GH in older women can upregulate expression and activity of GHRs, beneficial to improving the mitochondrial function, quality of oocytes and fertilization rate (54).

Effect of GH on Endometrial Receptivity

Good endometrial receptivity is the precondition for embryo implantation. During the treatment process of ovulation promotion with IVF, supra-physiological levels of estrogen regulate effects of endogenous hormones on endometrial thickness and pattern and expression of receptors and related factors to subsequently affect the endometrial receptivity (55). The uterus produces GH, which in turn adjusts the uterus (6), increases endometrial blood flow and expression of related cytokines and subsequently improves the endometrial receptivity (56). GH can also promote expression of endometrial vascular endothelial growth factor (VEGF)-1, leukemia necrosis factor, and matrix metalloproteinase 9, resulting in proliferation of endometrial glands, expansion of glandular cavity, blood vessel formation, and differentiation, thickening of endometria and endometrial mesenchyme (5, 57). The endometrial receptivity is consequently improved. In addition, GH can increase synthesis of IGF-I in the ovary, enable the pituitary gland to secrete more FSH and promote secreting function of the granulosa cells so as to increase the estrogen level for improving the endometrial thickness and pattern.

APPLICATION OF GH IN IVF

In 1988, Homberg et al. (58) found that GH increases the ovary sensitivity to the ovulation-inducing effect of gonadotropins, with significant reduction in the amount, duration of treatment and daily effective dose of human menopausal gonadotropin caused by GH addition. In an randomized clinical trial, Owen et al. demonstrated that GH improved the ovarian response to conventional ovarian stimulation regimens in females with poor ovarian responses, with significantly ($P < 0.05$) more follicles and more oocytes obtained in patients with polycystic ovaries when GH (24 IU) was administered on alternate days concurrently with the gonadotrophin treatment after enrollment

(59). Despite numerous studies, GH supplementation to the IVF regimen of poor ovarian responders remains controversial (60). Some studies suggested that pretreatment of GH could increase ovarian response to gonadotropins, improve oocyte quality and consequently be applied in the pituitary down-regulation cycle or in poor ovarian response to gonadotropins in the IVF (16, 61–64). Other authors did not support GH as an effective adjuvant for infertility treatment because the live birth rate was not increased even though some benefits might have been achieved through the use of GH (62, 65–67). However, some authors performed large-scale meta-analyses which supported GH as a useful *in vivo* adjuvant for human protocols (9, 10, 68). After analyzing six randomized controlled trials in a meta-analysis, Kolibianakis et al. found that GH addition significantly increased the clinical pregnancy rate by 16% (95% CI 4–28), the live birth rate by 17% (95% CI 5–30) and the proportion of patients reaching embryo transfer by 22% (95% CI 7–36) in poor-responding patients undergoing ovarian stimulation for IVF (10). Duffy et al. also performed a meta-analysis including ten randomized controlled trials to evaluate effectiveness of adjuvant GH in poor responders in IVF (9), and they found that a statistically significant difference in both the live birth rate (OR 5.39, 95% CI 1.89–15.35) and pregnancy rate (OR 3.28, 95% CI 1.74–6.20) favoring the use of adjuvant GH in IVF protocols for women considered poor responders without increasing adverse events. In another systematic review and meta-analysis including 22 eligible randomized controlled trials assessing interventions to improve the pregnancy rate in poor responders undergoing IVF (68), it was found that the only interventions that appear to increase the probability of pregnancy were the addition of GH to ovarian stimulation (OR for live birth: 5.22, 95% CI 1.09–24.99) and the performance of embryo transfer on day 2 compared with day 3 (the pregnancy rate was increased by 11.4%, 95% CI: 1.6–21.0). However, some randomized controlled trials discouraged the use of GH in IVF because no definitive benefits have been demonstrated in increasing the live birth rate for poor responders (18, 19), but careful evaluation of these trials showed severe drawbacks as stated before. Up to now, GH has been widely applied in the reproduction area but primarily for poor ovarian responders (7, 8, 10, 11, 14, 16, 19, 60–63, 67–76), poor quality of embryos (16, 17, 67, 70, 74, 77), improper endometrial reaction (5, 12, 56, 78, 79) and repeated failure of embryo transfer (1, 63, 80–82).

GH Application for Improving Ovarian Response

GH application combined with gonadotropins for ovulation promotion can improve the pregnancy outcome in most patients with poor ovarian responses. Lattes et al. (16) performed a prospective controlled study in 64 poor responders to previous IVF cycles who failed to achieve clinical pregnancy and were supplemented with low-dose GH in a subsequent cycle with the same gonadotropin dose and protocol. It was found that daily administration of low-dose (0.5 IU) GH from the first day of the GnRH agonist until the day of hCG application could significantly increase the clinical pregnancy rate (34.4 vs. 0%), number of both top quality embryos (1.03 ± 1.17 vs. 0.64 ± 0.88, $P = 0.046$) and cryopreserved embryos (0.85 ± 1.49 vs. 0.30 ± 0.81, $P = 0.02$). In a prospective controlled trial investigating the efficacy of GH pretreatment within an antagonist protocol in IVF/ICSI (intracytoplasmic sperm injection) cycles in poor responders, use of low-dose (4IU/d) GH on the start of ovarian stimulation could significantly decrease the effective dose of gonadotropins (median 750, low quantile (LQ) 533.3 and upper quantile (UQ) 1312.5 for GH group vs. 1375, 862.5, and 2962.5 for non-GH group, respectively) and duration of stimulation (median 8d, LQ 7d and UQ 10 d for GH group vs. 9d, 8d, and 10d for non-GH group, respectively), but increase the total number of oocytes (median 4, LQ 3 and UQ 7 for GH group vs. 3, 2 and 4 for non-GH group, respectively), metaphase II stage oocytes (median 2, LQ 1 and UQ 6 for GH group vs. 1, 0 and 2 for non-GH group, respectively), two pronucleus zygotes (median 2, LQ 0 and UQ 3 for GH group vs. 1, 1 and 2 for non-GH group) and good-quality transferred embryos (median 1.5, LQ 1 and UQ 2 for GH group vs. 0, 0 and 1 for non-GH group, respectively), with ultimate increase in the clinical pregnancy rate (21.74 vs. 0%) (74). A meta-analysis studying the influence of different GH addition protocols to poor ovarian responders on clinical outcomes in controlled ovary stimulation cycles demonstrated that either high- (12IU/d or 24 IU/qod) or low-dose (2IU/qod) GH could significantly improve the clinical pregnancy (RR 1.76, 1.25–2.48) and the live birth rate (RR 1.91, 1.29–2.83) in poor ovarian responders even though GH supplementation in the middle of luteal phase did not increase the pregnancy and live birth rates (11). In a prospective randomized clinical trial investigating GH as an adjuvant therapy added to either long or short agonist protocol, miniflare or antagonist protocols with GH introduced on day 6 of human menopausal gonadotropin stimulation in a dose of 2.5 mg S.C. daily till the day of hCG administration, the long GH agonist protocol was superior to the other three protocols regarding the number of oocytes retrieved (5.06 ± 1.83 vs. 4 ± 1.69, 4.95 ± 1.9 and 4.98 ± 2.15) and fertilized (3.73 ± 1.47 vs. 3.02 ± 1.51, 2.89 ± 1.14 and 3.57 ± 1.41) (83). But the clinical pregnancy rate was not significantly different among the four different protocols (36.7 vs. 23.2, 25.9, and 30.4%, $P > 0.05$) even though there was a difference in favor of the long GH agonist. Since the long GH agonist protocol required significantly greater gonadotropin dose and longer duration of stimulation, low-dose GH was suggested for GH supplementation protocol because low-dose GH could improve the reactivity of the ovary.

GH Application for Improving Oocyte Quality

In a sequential crossover study of IVF to evaluate GH supplementation in poor-prognosis patients based on the past failure to conceive due to low response to high-dose stimulation (<3 metaphase II oocytes) or poor-quality embryos (17), GH supplementation (10 IU) could significantly improve the clinical pregnancy rate (20 vs. 9%, $P < 0.05$) per fresh transfer and per frozen-thawed embryo derived from GH cycles leading to a highly significant productivity rate (30 vs. 14%, $P < 0.001$). These

GH effects were significant across all age groups, especially in younger patients (24 vs. 10% for patients <35 years but 15 vs. 11% for >40 years), and independent of stimulation modality or number of transfers. In this study (17), GH (10 IU) was injected in the previous cycle on days 7, 14 and 21 with a final injection on day 2 of the treatment cycle for the first 4 years of the study, and for the last 2 years of the study, patients received six injections with the first beginning on day 21 of the preceding cycle and the subsequent injections being on days 2, 6, 8, 10 and 12 at the dose of 10 IU. Nonetheless, it was suggested that longer pretreatment (4–6 weeks before gonadotropin start) with low physiological dose of 2 IU/d GH might be more beneficial to follicular growth and development. GH is beneficial to the repair of oocytes and quality improvement of ova in older patients because it can upregulate expression of IGF-I in the ovary and stimulate production of oocyte-derived growth and differentiation factor and bone morphogenetic protein-15 (84). After studying the outcomes of poor responders following GH pretreatment (4.5 IU GH administered once every 2 days since day 16 of the previous cycle for six times and once every 2 days since stimulation day 1 for three times) with IVF/ICSI mild stimulation protocol (100 mg Clomiphene citrate administered daily from day two or three of menstrual cycle) in a retrospective analysis of 132 patients whose data were prospectively enrolled and maintained, Chu et al found that GH supplementation could significantly increase the good-quality embryo rate in either IVF (68.1 vs. 51.5%, $P = 0.008$) or ICSI (53.9 vs. 36.7%, $P = 0.045$) group (61). In an observational study investigating GH adjuvant therapy in patients with three or more IVF failures (15), GH supplementation in the dose of 8 IU administered from day 1 of stimulation until the trigger day could significantly increase the pregnancy rate (25.7 vs. 18.2%, $P < 0.01$) per retrieval in these patients, with the pregnancy rate being elevated to a level similar to that observed in the study center for the whole population. An improvement of cytoplasmic competence is proposed as an explanation for this.

GH Use for Improving the Uterine Receptivity

Adequate thickness of the endometrium is the key to successful implantation, and a thin endometrium is critical in implantation failure (85–87). A thin endometrium may be caused by impaired endometrial growth which is closely related to angiogenesis and uterine blood flow. Angiogenesis is necessary for endometrial growth following menstruation and can provide a vascularized receptive endometrium for implantation (88, 89). Uterine blood flow is also an important factor for endometrial growth and is closely related to endometrial vascular development (86, 90, 91). Low uterine blood flow may cause a decreased pregnancy rate in patients with IVF-ET (embryo transfer), suggesting a close relationship of uterine blood flow with uterine receptivity (92, 93). High blood flow impedance of the uterine and radial arteries, poor growth of glandular epithelium, decreased VEGF and poor vascular development have all been confirmed to be characteristic of a thin endometrium (86). High blood flow impedance in the uterine and radial arteries may impair glandular epithelium growth and decrease endometrial VEGF level, and low VEGF level may result in poor vascular development, further decreasing the endometrial blood flow. This is a vicious circle which may lead to a thin endometrium associated with poor endometrial receptivity. In a randomized controlled trial investigating effects of GH on uterine receptivity in women with repeated implantation failure in an oocyte donation program, it was demonstrated that administration of GH (dose and timing were not mentioned in the study) could significantly ($P < 0.05$) increase the endometrial thickness (9.3 $\pm$ 1.5 vs. 8.6 $\pm$ 1.0 mm), implantation rate), pregnancy (54.3 vs. 17.1% with the OR of 6.9 and 95% CI 2.2–22.5) and live birth rates (51.4 vs. 17.1%, with the OR 6.4 and 95% CI of 2.0–20.9), with no abnormality detected in any of the babies born (1). Consistent with the above results, the randomized controlled trial by Bassiouny et al also proved the protective effects of GH on the endometrial thickness during IVF (8), and in this trial with 141 patients randomized into two groups for GH or not, GH was administered on day 6 of human menopausal gonadotropin stimulation in a daily dose of 2.5 mg SC (equivalent to 7.5 IU) until the day of hCG triggering and significantly ($P < 0.05$) increased the endometrial thickness (12.14 $\pm$ 1.25 mm vs. 11.56 $\pm$ 1.56 mm) besides significant increase in number of collected oocytes (7.58 $\pm$ 1.40 vs. 4.90 $\pm$ 1.78), number of MII oocytes (4.53 $\pm$ 1.29 vs. 2.53 $\pm$ 1.18), number of fertilized oocytes (4.04 $\pm$ 0.96 vs. 2.42 $\pm$ 1.03) and number of transferred embruyos (2.89 $\pm$ 0.45 vs. 2.03 $\pm$ 0.82). After investigating the effects of GH on clinical outcomes following frozen-thawed embryo transfer in a prospective controlled study with 4 IU GH injected subcutaneously daily from day three of the menstrual cycle until the day of progesterone injection, Wang et al. (56) found that addition of GH could significantly ($P < 0.05$) increase the clinical pregnancy (49.4 vs. 32.5%), embryo implantation (22.7 vs. 14.3%) and live birth (41.6 vs. 24.7%) rate. The serum levels of estradiol (798.73 $\pm$ 654.13 vs. 602.32 $\pm$ 438.9 pmol/L) and IGF-1 (25.55 $\pm$ 2.87 vs. 24.37 $\pm$ 3.06 nmol/L), endometrial thickness (9.6 $\pm$ 1.0 vs. 9.2 $\pm$ 0.8 mm) and serum level of VEGF (251.03 $\pm$ 39.48 vs. 227.93 $\pm$ 36.94 ng/L) were also significantly ($P < 0.05$) increased by addition of GH (56). All these effects may be caused by GH to increase the endometrial blood perfusion and expression of cytokines related to endometrial receptivity. After investigating effects of GH on pregnancy rate of patients with thin endometrium in a randomized controlled study with GH administered in a daily subcutaneous injection dose of 5 IU on day 3 of their cycles until the 18th day, Cui et al. (5) found that GH treatment could significantly ($P < 0.05$) increase the endometrium thickness on day 3 (7.87 $\pm$ 0.72 vs. 6.34 $\pm$ 0.86), the implantation rate (24.4 vs. 10.5%) and clinical pregnancy rate (42.5 vs. 18.9%) compared with the control group. Moreover, administration of GH significantly up-regulated expression of VEGF, integrin beta 3 and IGF-I at both mRNA and protein levels (5). The integrin beta 3 is a generally accepted biomarker of uterine receptivity (94) and is decreased in patients with unexplained infertility, endometriosis, luteal deficiency and lower pregnancy rates (95, 96). Increase of integrin beta 3 in GH-treated patients provides evidence that GH has a positive effect on the improvement of endometrial receptivity and pregnancy outcomes (1, 5).

ADMINISTRATION PROTOCOL OF GH

Currently, the addition protocols of GH for poor ovarian responders in IVF include addition at 4–6 weeks before hCG administration, in the luteal phase of the preceding menstrual cycle (in the pituitary down-regulation phase within a gonadotropin-releasing hormone agonist long protocol), at the time of hCG administration, and at the middle and late follicular phases. The dose for GH is from 0.5IU/d to 12 IU/d, but a small dose was preferred in a recent study (3-6 IU/week) (7).

4–6 Weeks Before hCG Administration

Because this is at the antral follicle stage, the GH protocol should be of small doses with a long course of treatment. In a single-center retrospective study of GH supplementation in IVF patients classified as poor prognosis, Kevin et al. (77) treated these patients with GH administration on days 2–3 during the preceding menstrual cycle by hypodermic injection in the dose of 1.5 IU/d for 6 weeks prior to trigger with hCG. It was demonstrated that GH could significantly increase the implantation, clinical pregnancy by 3.42 fold (95% CI 1.82 to 6.44, $p < 0.0005$) and live birth rates by 6.16 fold (95% CI 2.83 to 13.39, $p < 0.0005$) despite these patients being significantly older with lower ovarian reserve than the control group. Cui et al. (5) also treated patients with thin endometrium by injecting GH (5 IU) subcutaneously on day 3 of their cycles until the 18th day, and the endometrium thickness (7.87 ± 0.72 vs. 6.34 ± 0.86), implantation (24.4 vs. 10.5%) and clinical pregnancy (42.5 vs. 18.9%) rates were all significantly increased compared with the control group.

At the Luteal Phase of the Preceding Menstrual Cycle

GH is mostly administered in the pituitary down-regulation phase for a gonadotropin-releasing hormone agonist long protocol at this stage. In a randomized prospective clinical trial investigating GH co-treatment within a gonadotropin-releasing hormone agonist long protocol in patients with poor ovarian response (14), poor responding women achieved more oocytes (6.5 ± 2.1 vs. 3.2 ± 1.4, $P < 0.001$) and higher fertilization rates (4.4 ± 1.8 vs. 1.5 ± 0.9) with decreased doses and duration of gonadotropin when GH was added in the pituitary down-regulation in the dose of 12 IU/d until the day of hCG. However, use of low-dose GH of 0.5IU/d until the hCG day in the luteal phase for pituitary down-regulation in a prospective self-controlled study could achieve a greater number of top quality embryos (1.03 ± 1.17 vs. 0.64 ± 0.88) and cryopreserved embryos (0.85 ± 1.49 vs. 0.3 ± 0.81) as well as an increased clinical pregnancy rate (34.4% vs. 0) (16). Nonetheless, some other studies had also pointed out that GH supplementation in the middle luteal phase did not increase the clinical pregnancy and live birth rates (14, 62, 72, 73). One retrospective case-control study which used GH supplementation in the middle luteal phase in a daily dose of 3.33 mg of GH administered in subcutaneous injection (62) reported no improvement in IVF cycle outcomes with the similar clinical pregnancy rate (29 vs. 32 %, $P = 0.99$), mean number of mature oocytes retrieved (2.5 vs. 5.0, $P = 0.13$) and mean number of embryos available for cryopreservation (0 vs. 0). In one randomized prospective study by Kucuk et al. (14) which used GH in the daily subcutaneous injection dose of 4 mg (equivalent to 12 IU) from day 21 of preceding cycle along with GnRHa until the day of hCG, although more pregnancies (38.7 vs. 20%) and more clinical pregnancies (32.3 vs. 16.7%) with fetal heart activity have been achieved in the GH group compared with the control group, the difference did not reach the statistical significance ($P < 0.05$). In the randomized prospective study on 82 poor responders (72), patients in the GH group received daily injection of 4 IU GH from day 21 of previous cycle until the day of hCG injection, and the reproductive outcomes were similar ($P > 0.05$) in both groups with similar chemical pregnancy (15 vs. 14. 3%) and clinical pregnancy rate (12.5 vs. 11.2%).

At the Start of Gonadotropin

One study performed in China demonstrated that supplementation of GH in the dose of 4.5IU/d at the start of gonadotropin could significantly increase the number of oocytes collected, metaphase II and fertilized oocytes, and top quality embryos (97). Although the biochemical pregnancy rate (36.45%), clinical pregnancy rate (22.51%), implantation rate (18.32%) and live birth rate (19.1%) were all higher in the GH than the control group (32.63, 20.98, 18.18, and 15.85%, respectively), no significant difference ($P > 0.05$) existed. This study had 90 women in either the GH or the control group and was performed in a 2-year period, and enrollment of more patients with increased period of study may probably increase the statistical power with the significance level surpassing 0.05.

At the Middle and Late Follicular Phases

In two randomized prospective clinical trials investigating the effects of GH added to the antagonist protocol in the IVF/ICSI cycles for patients with poor ovarian responses, when the GH was added on the 6th day in the dose of 2.5 mg/d (equivalent to 7.5 IU/d) until the hCG day, the mean number of oocytes collected, metaphase II and fertilized oocytes, top quality oocytes and endometrial thickness were all significantly increased but the clinical pregnancy rate was not significantly improved (11, 62). When GH was given in a daily subcutaneous dose of 8 IU from day 7 of exogenous gonadotrophin administration till the day following hCG triggering in women older than 40 years (13), patients in the GH –treatment group received slightly more embryos per transfer compared to the placebo group (4.2 vs. 3.5, $P > 0.05$), but significantly ($P < 0.05$) higher clinical pregnancy rate (26 vs. 6%) and clinical implantation rate (6.2 vs. 1.7%). The delivery rate (22 vs. 4%) and live birth (5.2 vs. 1.1%) rate were also significantly higher in patients with GH supplementation compared with controls.

SUBJECTS AND BENEFITS OF GH TREATMENT

As stated before, GH adjuvant therapy was clinically widely used in poor ovarian responders (7, 8, 10, 11, 14, 16, 19, 60–63, 67–76), poor quality of embryos (16, 17, 67, 70, 74, 77), improper endometrial reaction (5, 12, 56, 78, 79) and

repeated implantation failure (1, 63, 80–82). When it is used in patients with repeat implantation failure which is defined as failure of pregnancy despite implantation of a high-quality embryo at least three times or of over 10 embryos on repeat implantation failure (1, 63, 80–82), the mechanism of this action is, as stated before, related to GH stimulating proliferation and differentiation of granulosa cells, increasing production of estradiol in both early and late follicular development for animal and human ovaries, enhancing effect of FSH on the development of ovarian follicles and improving endometrial thickness (82, 98–100). A recent randomized controlled clinical trial performed in China of GH co-treatment on controlled ovarian stimulation in normal ovarian response women showed significantly ($P < 0.05$) higher two pronuclei rate (33.92 vs. 30.92%) and higher quality embryo rate (63.4 vs. 59.33%) besides significantly increased number of embryos available (3.79 ± 2.74 vs. 2.90 ± 2.12, $P < 0.001$) and higher endometrial thickness on hCG day (11.96 ± 2.24 vs. 11.62 ± 2.81, $P = 0.036$) in 781 patients receiving GH of 1IU/4IU administered daily since day two of the previous cycle or day two in accordance with controlled ovarian stimulation until hCG trigger in comparison with the control group without GH adjuvant therapy (79). Among a total of seven systematic reviews and meta analyses found online (pubmed) up to 2019 investigating the effect of GH adjuvant therapy on poor responders undergoing IVF (9–11, 65, 67, 68, 101), only two meta analyses demonstrated no improvement in the liver birth rate (65) or the clinical pregnancy rate (OR 0.051, 95% CI −0.033 to 0.134, $P = 0.197$) (67). All the other five meta analyses showed that GH supplementation in IVF significantly increased the live birth rate with the OR 5.22 and 95% CI 1.09–24.99 in the study by Kyrou et al. reported in March 2009 (68), OR 6 and 95% CI 3–20 in the study by Kolibianakis et al presented in the November-December issue of 2009 (10), OR 5.39 and 95% CI 1.89–15.35 in the study by Duffy et al published in January 2010 (9), OR 2.96 and 95% CI 1.17–7.52 in the study by Jeve et al. published in the April-June issue of 2016 (101), and OR 1.91 and 95% CI 1.29–2.83 in the latest study by Li et al. presented in March 2017 (11). Although some individual randomized controlled studies did not reveal significant improvement in the live birth rate in GH supplementation in women undergoing IVF, these pooled data in most meta-analyses favored the use of GH for IVF because of the significantly increased live birth rate.

In conclusion, GH supplementation in the process of IVF might improve reactivity of ovary, endometrial receptivity, clinical pregnancy and live birth rates. Although GH is frequently used as an adjuvant in patients with poor ovarian response for ovulation promotion and in patients with repeated implantation failure for improving the endometrial receptivity, no clear standards have currently been set up for the indications, methods and dosages in clinical application, and more in-depth studies are consequently needed for appropriately addressing these issues.

AUTHOR CONTRIBUTIONS

Y-MX, G-MH, and B-LG contributed substantially to the following aspects:

1. Substantial contributions to the conception or design of the work, or the acquisition, analysis or interpretation of data for the work.
2. Drafting the work or revising it critically for important intellectual content.
3. Provide approval for publication of the content: G-MH and B-LG.
4. Agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Growth Hormone Promotes *in vitro* Maturation of Human Oocytes

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Increasing the success rate of *in vitro* maturation (IVM) for human oocytes has a major clinical significance. Previous studies have shown that growth hormone (GH) added into IVM medium could promote IVM of oocytes from non-human beings. However, few studies on systematic IVM for human oocytes with GH have been reported. Human germinal vesicle (GV) oocytes collected for IVM were cultured with different concentrations of GH to optimize the concentration. Metaphase II (MII) stage oocytes obtained from IVM were fertilized by intracytoplasmic sperm injection (ICSI). Maturation rate, fertilization rate, and blastocyst rate were assessed after IVM with or without GH. Furthermore, gene expression profiles were compared in oocytes between the two groups using single-cell RNA-seq. The optimal concentration of GH for IVM was 200 ng/ml, and the maturation rate of this group reached 70% which was double that of the control group (35%, $P = 0.004$). The fertilization rate (73.1 vs. 60.3%) and blastocyst rate (25.0 vs. 15.5%) both had an increasing trend in the GH group compared to controls. Single-cell RNA-Seq and real-time PCR data showed that GH could significantly enhance the expression of genes associated with meiotic progression and embryo development, such as *AURKA* (aurora kinase A, $P = 0.007$), *PDIA6* (protein disulfide isomerase family A member 6, $P = 0.007$), *LINGO2* (leucine rich repeat and Ig domain containing 2, $P = 0.007$), and *CENPJ* (centromere protein J, $P = 0.039$). Taken together, GH could promote maturation of human oocytes, probably through accelerating meiotic progression, balancing redox homeostasis of cellular environment, and promoting oocyte developmental competence.

Keywords: growth hormone, human oocyte, *in vitro* maturation, single-cell RNA-seq, *AURKA*, *PDIA6*, *LINGO2*, *CENPJ*

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INTRODUCTION

Oocyte *in vitro* maturation (IVM) is a patient-friendly and less expensive human assisted reproduction technology (ART). It can effectively decrease the risk of ovarian hyperstimulation syndrome (OHSS) due to its low dosage of gonadotropin administration, and can also simplify treatment procedures by avoiding frequent ultrasonography. This technology can be applied to patients of polycystic ovary syndrome (PCOS) who have high risk of OHSS, patients with gonadotrophin resistance, and patients who have contraindications for ovulation stimulants. In addition, IVM can be used in fertility preservation. However, owing to the low success rate and

reduced oocyte developmental potentiality compared to conventional *in vitro* fertilization (IVF), IVM is difficult to be widely adopted in clinical practice. Thus, improving the success rate by optimizing the IVM culture system has a major clinical significance.

Growth hormone (GH) as a classical and pleiotropic peptide hormone has been paid more attention for its administration in ovarian co-stimulation for infertile women. Among distinct groups of infertile patients, GH applied *in vivo* has been reported to make effects on assisted reproduction outcomes. For the patients who responded normally, the treatment of GH could raise the implantation rate and pregnancy rate (1). For poor ovarian responders, women of advanced age, and patients with multiple IVF failures, GH treatment could also improve the outcome of assisted conception by increasing the number of metaphase II (MII) stage oocytes, two-pronuclear zygotes (2PN) and transferred embryos (2–6). *In vivo* administration of GH has also been reported to promote IVM of human germinal vesicle (GV) stage oocytes (7).

In addition to application *in vivo*, the positive effects of GH added into IVM medium have been intensively investigated in many species of animals. Related studies showed that GH could promote oocytes maturation and embryo development in bovine (8–14), ovine (15), equine (16–19), porcine (16), rat (20), mouse (21), canine (22), and rhesus macaque (23). Its facilitatory role in IVM and embryo development of animals can enlighten us on the advancements of human IVM.

Few studies on systematic IVM for human oocytes with GH have been reported. To examine the effects of GH on human oocyte IVM, the immature oocytes derived from ICSI cycles could be made use of to perform a preliminary study. These oocytes are often discarded and considered to be useless. However, these immature oocytes have their own value. They can achieve maturation and early embryo development *in vitro* (24), and obtain pregnancy (25). If these oocytes could be rescued to maturation for clinical purposes, the patients may benefit from it. There has been a case report showing a successful pregnancy and delivery through one naked GV oocyte from stimulated ovary cultured to maturation with GH *in vitro* (26).

In this study, we gathered GV-stage oocytes from ICSI patients to identify whether GH works during human oocyte IVM and to clarify the optimal GH concentration. Then single-cell RNA-seq was employed to explore the mechanism of GH.

MATERIALS AND METHODS

Participants

The GV-stage oocytes were donated by patients who underwent ICSI treatment due to male factors at the Center for Reproductive Medicine, Shandong University.

Ethical Approval

The study was approved by the Institutional Review Board of Reproductive Medicine, Shandong University. Each participant in the study had written the informed consent. The blastocysts in this study, which were formed by both oocytes and sperms donated for research, would not be used for any reproductive purpose and were all destroyed after observation.

Oocytes Collection

Follicles were punctured under ultrasound guidance 36 h after the administration of 10,000 IU human chorionic gonadotropin (hCG, Merck Serono, Switzerland). The corona-cumulus cells were removed by hyaluronidase (Irvine Scientific, USA), and then the meiotic status of human oocytes was assessed. Only MII-stage oocytes were fertilized by ICSI for the patients. Those GV-stage oocytes with a discernable germinal vesicle were donated and collected for this study.

IVM and ICSI

The GV-stage oocytes obtained in each experimental day were randomly distributed to different concentration groups including one control group, and we always cultured fresh GV oocytes. An accumulating total of 252 GV-stage oocytes were made use of and divided into eight groups with different concentrations of GH. The concentration gradients were set as 0 (control), 10, 50, 100, 200, 300, 500, 1,000ng/ml (8, 16, 27). They were cultured for 24 h in the IVM medium, which was Medium 199 (Gibco/life technologies, USA) and meanwhile supplemented with 0.29 mmol/L sodium pyruvate (Sigma, USA), 10% human serum albumin (Vitrolife, Sweden), 0.075 IU/mL recombinant follicle stimulating hormone (FSH, Merck Serono, Switzerland), 0.15 IU/mL hCG, and 10 ng/mL epidermal growth factor (EGF, Sigma, USA). Then the number of MII-stage oocytes were counted, and the criterion of nuclear maturation was the extrusion of the first polar body. Then the sperms donated for research were used to perform ICSI for MII-stage oocytes obtained through IVM. ICSI was carried out under an inverted microscope (Nikon, Japan), and all procedures employed sequential culture media supplied by Vitrolife (G-IVF, G1 and G2, Sweden). 16 to 18 hours after ICSI, the conditions of fertilization were observed and the number of two pronuclear zygotes (2PN) was counted. Five to six days after fertilization, the number of blastocysts was determined. All embryos were incubated up to the blastocyst stage according to Gardner's criteria (28). The fertilization rate was the percentage of number of 2PN accounting for number of MII oocytes. The blastocyst rate was the percentage of number of blastocysts accounting for number of MII oocytes.

Oocyte RNA Sequencing

Three pairs of GV-stage oocytes from three patients respectively were cultured for 24 h with 200 ng/ml GH or not and then washed with phosphate-buffered saline (PBS). Each oocyte was collected and transferred into a 0.2 ml RNase-free microcentrifuge tube containing 2 μl lysis buffer, which was composed of 0.2% Triton X-100 (Sigma, USA) and 2 U/μl of RNase inhibitor (Clontech, USA). Then the cDNA was obtained by the Smart-Seq 2 method (29). For each sample, 20 ng cDNA was used for the Library construction. Then the constructed Library was sequenced on the platform of Illumina HiSeq.

Sequencing Data Analyses

By using the sequencing strategy of PE150, pair-end reads were obtained. The software of fastqc was used for the quality control analysis of the reads. Using Tophat2, the reads were aligned to reference genome, which was downloaded from the Ensemble database. After that, the software of RseQC was used to do the quality control for the alignment data. According to the reads alignment results, reads were assigned to specific transcripts to count the transcript reads. TPM (Transcripts per Million) was adopted and used for standardization. Reads were aligned to mRNA sequences by the bowtie software and the mRNA quantitative analyses were done using eXpress software. Differential Expression Analysis of mRNA was performed by DESeq software. In the case of biological duplication, differentially expressed mRNA was filtered by *P*-value and adjusted *P*-value, which was the *P*-value after multiple comparison correction.

Real-Time PCR for Validation

The sequencing results were validated by real-time PCR using nine pairs of oocytes from nine patients, respectively. The cDNA from these 18 oocytes was obtained through REPLI-g WTA Single Cell Kit (Qiagen, Germany) and was used for real-time PCR. Real-time PCR was performed with SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio Inc., Japan) in the LightCycler 480 II (Roche, Germany). Gene specific primers were designed by Primer Premier 5.0 Software (Premier Inc., Canada; **Supplementary Table S1**). The relative expression level of these genes was normalized by the housekeeping gene of *GAPDH*.

Statistical Analysis

Categorical data were displayed as counts and percentages and analyzed by chi-square test. Real-time PCR data were counted and analyzed using the method of $2^{-\Delta CT}$ (30), expressed as the mean ± SEM, and compared by paired-samples *t*-test with two tails. Statistical analyses were performed with SPSS software, and statistical significance was considered as $P < 0.05$.

RESULTS

Optimal Concentration of GH for Human Oocyte IVM

As shown in **Table 1**, a total of 252 GV-stage oocytes were cultured in eight groups with different GH concentrations. After IVM, the maturation rate of control group was 35%, while in 200 ng/ml GH group, the maturation rate was the highest (70%, $P = 0.004$).

GH May Have an Effect on Fertilization and Early Embryo Development

After the optimal GH concentration for IVM was confirmed, MII-stage oocytes ($N = 52$) cultured with 200 ng/ml GH and MII-stage oocytes ($N = 58$) from the control group were collected and fertilized by ICSI. The fertilization rate of the GH group was 73.1%, which was higher than 60.3% of the control group ($P = 0.158$). The blastocyst rate of the GH group was 25.0%, which was higher than 15.5% of the control group ($P = 0.214$). But these two differences were both of no statistical significance (**Table 2**).

TABLE 1 | Maturation rates of IVM according to different GH concentrations.

Groups (GH concentration ng/ml)	No. of GV oocytes	No. of MII oocytes (24 h)	Maturation rate (%)
0	40	14	35.0[a]
10	30	15	50.0
50	31	18	58.1
100	31	17	54.8
200	30	21	70.0[a]
300	30	20	66.7
500	30	17	56.7
1,000	30	15	50.0

[a] *P < 0.05.*

TABLE 2 | Fertilization rates and blastocyst rates between GH and the control group.

	Control	GH (200 ng/ml)	*P*-value
No. of MII	58	52	
No. of 2PN (fertilization rate)	35 (60.3%)	38 (73.1%)	0.158
No. of blastocysts (blastocyst rate)	9 (15.5%)	13 (25.0%)	0.214

2PN represents the two-pronuclear zygote.
Fertilization rate is based on No. of 2PN/No. of MII.
Blastocyst rate is based on No. of blastocysts/No. of MII.

Distinct Gene Expression Profiles in GH Cultured Oocytes

By PCA (principal component analysis) for the three pairs of oocytes after RNA sequencing, we found one oocyte in the control group behaved differently compared to another two. Therefore, we only made use of the sequencing results from two pairs of oocytes. The MA plot showed that there were 295 up-regulated and 212 down-regulated transcripts which represented the corresponding genes according to adjusted *P*-value < 0.05 (**Figure 1**). In the heatmap, the control group samples and the experiment group samples were clustered respectively, which showed the consistency between the same kind samples. Dramatic changes of gene expression levels between the two groups were also revealed by the heatmap (**Figure 2**).

Real-Time PCR for Validation

In order to get the most significantly different genes between the two group, we filtrated the genes according to adjusted *P*-value < 0.001. The gene set was exhibited in **Supplementary Table S2**. Five genes were validated successfully in accordance with the sequencing results. They were as follows: *AURKA* (aurora kinase A), *CENPE* (centromere protein E), *PDIA6* (protein disulfide isomerase family A member 6), *LINGO2* (leucine rich repeat and Ig domain containing 2), and *CENPJ* (centromere protein J). Nine pairs of GV-stage oocytes from nine cases respectively

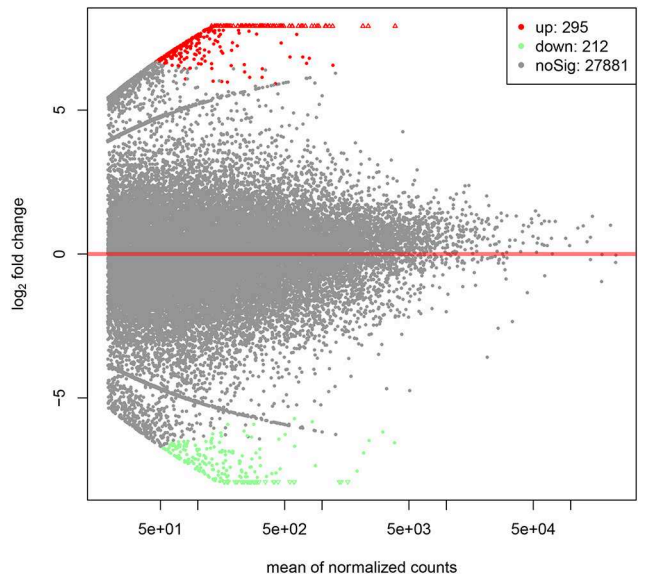

FIGURE 1 | The MA plot of distinct gene expression profiles in GH cultured oocytes compared to the control group according to adjusted *P*-value < 0.05. X-axis represents the mean of normalized counts between the two groups; Y-axis represents the logarithm of fold change. From left to right in the X-axis, the expression level of the genes is from low to high. In the Y-axis, the more deviation it is from Y = 0, the larger the fold change is.

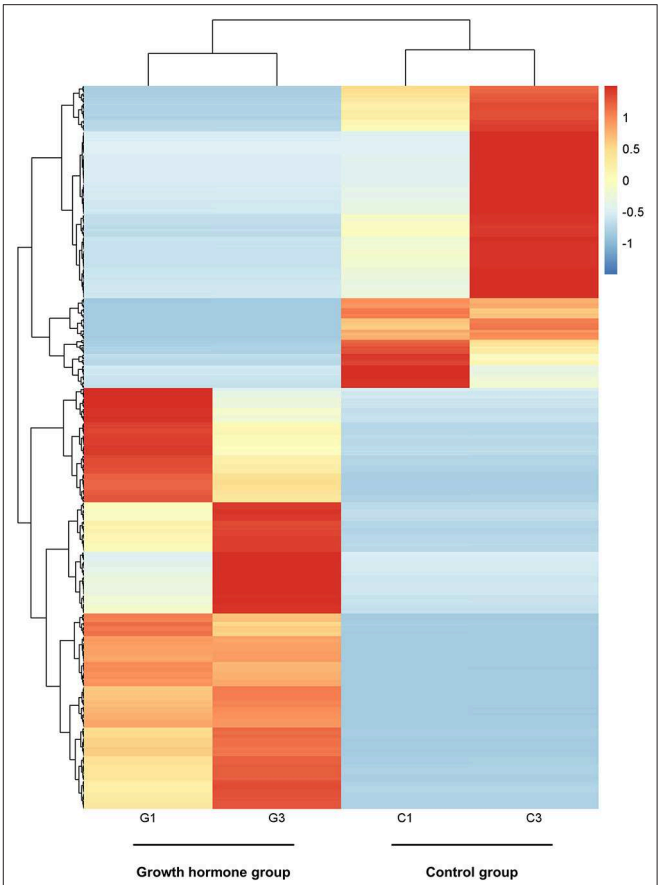

FIGURE 2 | The heatmap of distinct gene expression profiles in GH cultured oocytes compared to the control group according to adjusted *P*-value < 0.05. Each row represents one gene expression levels among the four samples; Each column represents the expression levels of the genes in one sample; The black tree lines represent the results of layered clustering for the genes or samples, and the similar ones will be clustered. The different colors represent different expression levels and the illustration in the right represents the corresponding relationship between colors and the value of expression levels after standardization. G1and G3 represent samples from GH group; C1 and C3 represent samples from the control group.

were collected for *in vitro* culture with 200 ng/ml GH or not. After 24 h for IVM, the cDNA of these oocytes was obtained for validation, the results were displayed in **Figure 3**. The relative gene expression levels of *AURKA* (2.1-fold, $P = 0.007$), *PDIA6* (2.5-fold, $P = 0.007$), *LINGO2* (5.5-fold, $P = 0.007$), and *CENPJ* (1.9-fold, $P = 0.039$) were all significantly higher in the GH group compared to the control, and *CENPE* (3.5-fold, $P = 0.098$) tended to be increased by GH.

DISCUSSION

The clinical application of IVM is limited by its low success rate. In the present study, we found that GH could promote human oocyte maturation *in vitro*, and the concentration was optimized. We compared transcriptome profiles of human oocytes matured *in vitro* with GH or not by single-cell RNA-seq, which suggested that GH probably work by accelerating meiotic progression, balancing redox homeostasis of cellular environment, and promoting the oocyte developmental competence.

Building an IVM culture system with a higher success rate by adding effective ingredients has its clinical importance. In addition to the above-mentioned case report (26), it has only been reported that 1,000 ng/ml GH had no effect on IVM of human GV-stage oocytes, which were obtained during gynecologic surgery (31). Few studies have explored the optimal concentration of GH for human oocyte IVM. By increasing the concentration of GH gradually, we found that when it reached 200 ng/ml, the maturation rate reached the maximum. If the GH concentration was increased continually, instead, the maturation rate went down. Obviously, the 200 ng/ml was the optimal concentration. In this study, the maturation rate of IVM in the control group was 35%, which was close to the literature report of 38% (32). The maturation rate of 200 ng/ml GH group reached 70% which was double of the control group, and this indicated that GH could remarkably promote nucleus maturation of human oocytes. Furthermore, there have been studies identifying the presence of GH receptor on human oocytes (33, 34), and this may underlie the fact in this study that GH works in naked oocytes. The fertilization rate (from 60.3 to 73.1%) and blastocyst rate (from 15.5 to 25.0%) both had a tendency to be up-regulated by 200 ng/ml GH. No significant difference might be attributed to the sample size. This revealed that GH might have benefits on fertilization and early embryo development *in vitro*.

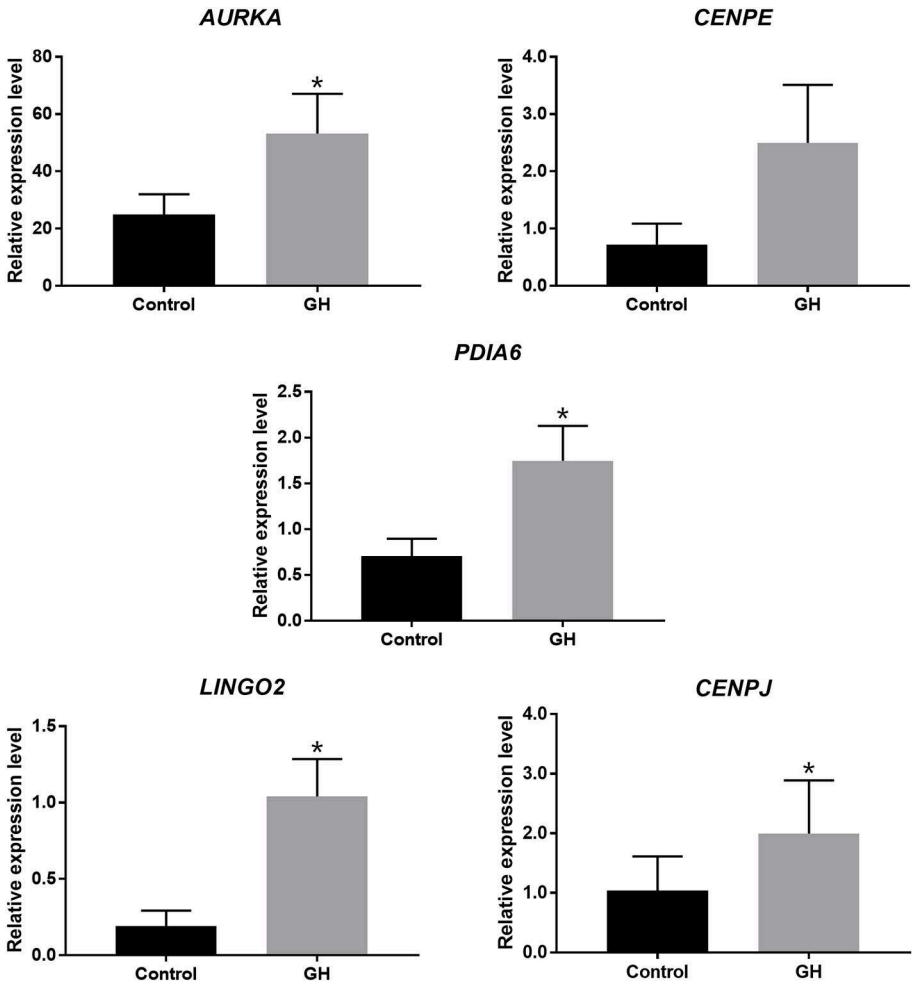

FIGURE 3 | Real-time PCR for validation. *$P < 0.05$.

We further explored the roles of GH by single oocyte RNA-Seq. *AURKA* (aurora kinase A) in the GH group was 2.1-fold higher than the control with statistical significance. The intracellular localization of Aurora A is at the meiotic spindle poles and at a contractile ring/midbody during the first polar body extrusion, and a role in microtubule assembly and spindle organization is indicated (35–37). It has been reported that in *Xenopus* oocyte, the overexpression of Aurora A accelerated progesterone-induced GVBD (germinal vesicle breakdown) (38). In mouse oocyte, when Aurora A antibody was microinjected, the rate of GVBD was decreased (36). In porcine oocyte, Aurora A promoted the meiotic resumption (39). *CENPE* (centromere protein E) had a 3.5-fold increase in the GH group of this study. CENP-E is an essential meiotic kinetochore motor, and is required for meiotic progression; When mouse GV oocytes were injected with anti-CENP-E antibody, >95% of oocytes were arrested at MI even after 24 h, failing to extrude the first polar body (40). Therefore, in this study, GH might work through accelerating meiotic progression.

PDIA6 (protein disulfide isomerase family A member 6) of GH group was significantly 2.5-fold higher than the control. *PDIA6* is a redox gene associated with redox homeostasis, and the expression of *PDIA6* was downregulated in *in vitro*-matured MII oocytes compared to *in vivo*-matured MII oocytes for both prepubertal and adult pigs (41). As we know, the *in vivo* maturation environment is more complete and more effective than *in vitro* for oocytes. In this study, *PDIA6* expression was up-regulated in the GH group, and this revealed that GH addition made the culture environment better for oocyte to balance the redox homeostasis.

LINGO2 (leucine rich repeat and Ig domain containing 2) was remarkably increased by GH to 5.5-fold of the control. It has been reported that the expression level of the *Lingo2* gene increased gradually as the mouse embryo developed (42). This indicated that the more fully the embryo developed, the higher the expression level of *Lingo2* gene was. The expression level of *CENPJ* (centromere protein J) in the GH group was significantly up-regulated to 1.9-fold of the control. CENPJ is required for the biogenesis of centrioles, which organize centrosomes. For animal cells, centrosomes are the microtubule-organizing centers (43). What's more, centrosomes have an effect on cell polarity establishment, organelles positioning, and

cell division organization (44). CENPJ is essential for centriole formation and defective centriole formation results in aberrant spindle positioning (45). While in the oocytes of most metazoan species, the centrioles and centrosomes are lacking (46). The zygotic centrosome will be restored at fertilization, and the functional zygote centrosome requires the blending of maternal centrosomal proteins and paternal reproducing element (47). The female cytosolic factors involved in the reformation of zygotic centrosomes are generated during oocyte meiosis in the preparation for fertilization, and the expression of centrosomal proteins in oocytes has already risen in meiosis II, just before fertilization (48). Thus, the expression of *CENPJ* in mature oocytes probably offers the preparation for centrosome restoring in zygote. In this study, by up-regulating *CENPJ* and *LINGO2*, GH might promote the developmental competence of the oocytes, and enable oocyte to reserve more useful materials in preparation for later fertilization and embryo development.

Taken together, this study identified that the optimal GH concentration 200 ng/ml could increase the success rate of human oocyte IVM. GH might play its roles by up-regulating *AURKA, PDIA6, LINGO2,* and *CENPJ,* which probably work through accelerating meiotic progression, balancing redox homeostasis of cellular environment, and promoting the oocyte developmental competence.

DATA AVAILABILITY

The single-cell RNA-seq data in this study have been deposited in the GEO database, and the accession number is GSE133161.

ETHICS STATEMENT

The study was approved by the Institutional Review Board of Reproductive Medicine, Shandong University. All human subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SZ designed the study. HZ and JM recruited the subjects. YL performed the experiments, analyzed the data, and wrote the paper. HL performed part of the experiments and analyzed the data. QY performed part of the experiments. H-BL and TH contributed to the reagents and materials, and assisted part of the experiments. SZ revised the manuscript and gave final approval of version to be published. All authors critically reviewed and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Growth Hormone and Endometrial Receptivity

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Administration of growth hormone (GH) during ovarian stimulation has shown beneficial effects on *in vitro* fertilization (IVF) outcomes. It is generally believed that this improvement is due to the stimulating effect of GH on oocyte quality. However, studies are emerging that show possible positive effect of GH administration on endometrial receptivity, thus suggesting an additional potential benefit at the level of the uterus, especially among women with recurrent implantation failure, thin endometrium, and older normal responders. This review summarizes recent data on GH co-treatment effects on endometrium and endometrial receptivity among infertile women undergoing IVF, and proposes possible mechanisms of GH actions in the endometrium.

Keywords: endometrium, endometrial receptivity, growth hormone, infertility, *in vitro* fertilization, transcriptome

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INTRODUCTION

Receptive endometrium is an absolute prerequisite for a successful embryo implantation, being defined by a limited time-frame when the endometrium is favorable for embryo adhesion and the subsequent attachment and invasion processes (1).

Endometrial receptivity is a complex process that is orchestrated by the synergistic actions of main reproductive hormones estrogen and progesterone, as well as plead of other endocrine, paracrine and autocrine factors (2, 3). Impaired endometrial receptivity is thought to be one of the major reasons for embryo implantation failure (4). In assisted reproductive technologies (ART), where the good quality embryos are transferred as a standard of care, implantation failure remains an unsolved obstacle (5, 6). Regardless of the advances in assisted reproduction, particularly regarding the more effective means of embryo selection and cryopreservation, many patients repeatedly fail the treatment procedure. What we are facing today is that implantation failure in ART is common, and we lack the evidence-based therapeutic solutions for treating it. As a result, clinicians often feel obliged to offer treatments that are largely empirical, based on some biologic rationale but with little clinical evidence to support their use (7, 8). The treatment failure is equally frustrating to both patients and their providers, which even more emphasizes the urgent need for novel effective treatment to prevent yet another failure.

The role of growth hormone (GH) in female reproduction has gained renewed interest and has become a heated topic over the last decade. The local GH production in the reproductive tissues themselves exert an important autocrine/intracrine effects on those tissues, in addition to the pituitary production of GH (9). Moreover, local insulin growth factor 1 (IGF-1) production (known downstream mediator of GH) has been shown to be controlled by gonadotropins and estradiol as well (10). Evidence emerging from clinical practice suggests that GH administration during ovarian stimulation may improve oocyte quality [higher number of oocytes collected, higher fertilization

rate, and higher number of embryos reaching the transfer stage (11–15)], increase pregnancy rate (16–24), implantation rate (16, 20–23, 25, 26), and live birth rate (12, 16, 19, 20, 23, 25, 27). The accumulating beneficial effects of GH on assisted reproduction outcomes do not exclude the possibility that this effect is due, at least in part, to an action of GH on endometrial receptivity.

GROWTH HORMONE IN THE ENDOMETRIUM

GH is a peptide hormone secreted by the anterior pituitary gland, having important role in cell growth and metabolism throughout the body. GH together with its receptor, GHR, and related growth factors including IGF-1, is expressed in the endometrium of rats and human (28–31). The study by Sbracia et al. obtained biopsies from women in proliferative and secretory phases, as well as first trimester decidua (from elective pregnancy terminations) (28). They showed that there was no GH expression in proliferative glandular epithelium, but GH immunoreactivity appeared in the mid-luteal secretory phase (no subdivision within secretory phase was done) and increased in the decidua from the first trimester abortions, with similar expression in the decidual samples from the term pregnancies, suggesting a role in embryo implantation process. Interestingly, no stromal expression of GH was observed in any sample (28). Moreover, the authors analyzed GH expression in the endometrium from women with "luteal phase defect," defined by low progesterone levels <8 ng/mL and delayed endometrial maturation, and saw significantly lower expression of GH (28). This data suggested close relationship between GH expression in endometrium and progesterone level/function. Further, a recent study on human endometrial cell line indicated that GH may act in a direct or IGF-1-mediated manner on human endometrial cells to promote proliferation and vascularization and up-regulation of receptivity-related genes such as vascular endothelial growth factor (VEGF) and integrin beta 3 (ITGB3) (21). VEGF is an important player in angiogenesis (32), and it has been shown to act in an autocrine manner on endometrial epithelial cell adhesion as a key regulator in the implantation process (33). ITGB3 is a well-known biomarker of receptivity (34), and down-regulation of this biomarker (phenomenon detected in women with unexplained infertility, endometriosis, and luteal phase deficiency) has been related to lower pregnancy rates (35, 36).

Apart from the effects of circulating GH and locally produced GH on endometrium, there is a proposed indirect effect of ovarian GH on endometrial function, namely its involvement in the function and maintenance of the corpus luteum (37, 38). While the majority of the data come from various animal models, they are nevertheless significant. Luteal function and its maintenance are vital for the establishment of pregnancy and its viability due to the production of progesterone by the corpus luteum—the main "keeper" of the normal early pregnancy. Hence, the stimulatory effect of GH on ovarian steroidogenic cell function may play a major role in endometrial function and dysfunction via its effect on ovary (see **Figure 1** for the proposed mechanisms of GH action on endometrium).

CLINICAL USE OF GH AND EFFECT ON THE ENDOMETRIUM

Initial reports on the use of GH in clinical practice come from cases of hypogonadotropic hypogonadism or panhypopituitarism (46). Subsequently, the use of GH has been expanded on different patient population, such as women with poor ovarian reserve, poor responders, or with poor oocyte quality due to advanced maternal age (25, 47, 48). In general, GH administration in the infertility clinic setting has focused on GH effects on oocyte, and the effect on endometrium has been largely overlooked.

Subsequently, the attention has been turned onto the endometrium, and interesting observations have been made suggesting positive effect of growth hormone treatment on endometrial thickness and implantation potential (see **Table 1** for the studies). A case report of a patient with panhypopituitarism demonstrated improved endometrial thickness and successful implantation and pregnancy after adding growth hormone to the treatment protocol following multiple failed *in vitro* fertilization (IVF)/embryo transfer cycles (55). Alternatively, a study on 20 patients with documented GH deficiency reported improved embryo quality, but no improvement in endometrial thickness, when supplemented with GH in IVF cycle (15) (**Table 1**). Below we will discuss the available literature on the use of GH in various clinical IVF settings.

Infertile Patients With Recurrent Implantation Failure

This is a group of patients that fail to achieve pregnancy in fresh or frozen embryo transfer cycles despite appropriate endometrial development (thickness and pattern) and good quality embryo transferred. Patients with recurrent implantation failure (RIF), having undergone three or more embryo transfer cycles after IVF treatment without a clinical pregnancy, are among the most difficult patients to treat, with no proven standard treatment. Impaired endometrial maturation is suggested as a common cause for RIF (56–58), making it a target patient group who could potentially benefit from GH co-administration during IVF procedure. Chen et al. study on 42 RIF patients undergoing IVF treatment found that GH treatment throughout the stimulation increased the endometrial thickness and consequent pregnancy and live birth rates among young patients <35 years old supplemented with GH when compared to no GH RIF group (19). Patients in both groups had similar peak estradiol levels and similar number of oocytes retrieved (19). While it is unclear if the difference in endometrial thickness of 11.61 ± 2.9 vs. 9.7 ± 1.46 mm between study and control groups, respectively was crucial in achieving higher pregnancy rates in the study group, the observation is nevertheless important. This has been reported again in the second study, a randomized clinical trial including 70 RIF patients in oocyte donation program (as an ideal model for assessing GH effect on patient's endometrium without

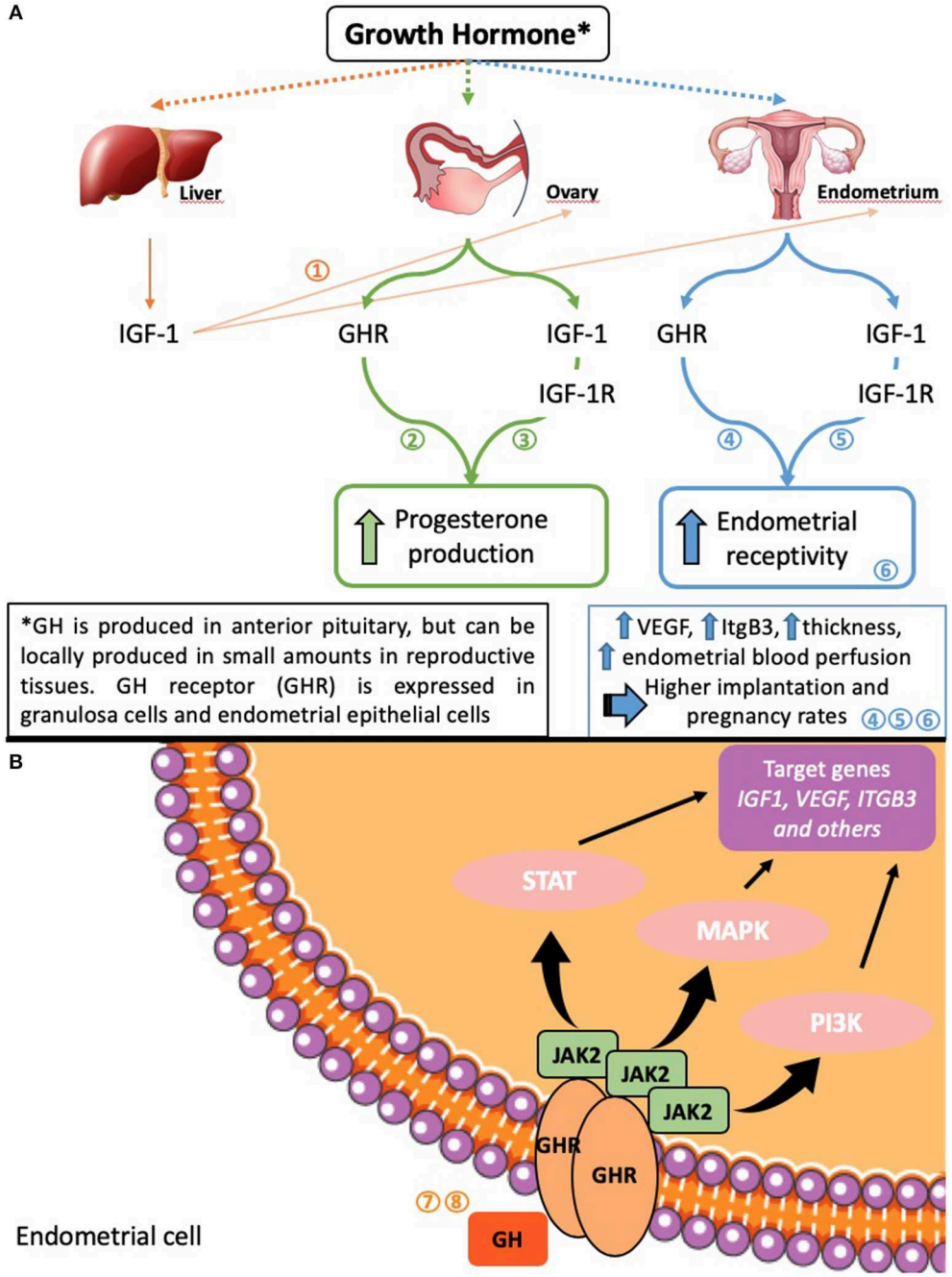

FIGURE 1 | Possible mechanisms of GH effects on ovarian and endometrial function **(A)** and on endometrial cells **(B)**. Numbers in the figure indicate studies where the information is presented in detail: 1 (39); 2 (40, 41); 3 (42); 4 (23); 5 (21); 6 (43); 7 (44); 8 (45).

confounding factors of ovarian age and response) (20). In that study patients, who were treated with GH throughout medicated frozen embryo transfer cycle demonstrated significantly thicker endometrium, 9.3 ± 1.5 mm vs. 8.6 ± 1.0 mm, respectively, and higher pregnancy and live birth rates compared with RIF patients in the placebo group (20) (**Table 1**).

These are the first two studies assessing GH effects on endometrium in RIF patients, and, although the findings are promising, clearly more studies on larger patient population, as well as randomized clinical trials (RCTs), are needed for any clinically meaningful conclusions. It is well-accepted that endometrial thickness does not necessarily mean that the endometrium is receptive, yet it is considered as a measure of endometrial maturity, and optimal growth of the endometrium (>7 mm) is required for a successful embryo implantation (59–61).

TABLE 1 | Studies assessing the effect of growth hormone (GH) co-treatment in *in vitro* fertilization (fresh treatment cycles and frozen embryo transfer cycles) on endometrium.

Study	RCT	Study group; Ethnicity	GH/control (mean age)	Inclusion criteria	Exclusion criteria	Intervention	Primary outcome	Effect on endometrial thickness (mm)		
								GH	Control	*p*-value
FRESH EMBRYO TRANSFER CYCLE										
Rajesh et al. (15)	No	Infertile women with GH deficiency; Chinese	20/20* (32.9 y) *same women cycle before without GH served as controls	GH deficiency based on clonidine test; previous IVF cycle without GH; became pregnant with GH treated cycle	Panhypopituitarism; GH deficient patients with previous cycle treated at other hospital	12 IU GH every 3rd day, starting from GnRH stimulation day until hCG administration	Improved embryo quality; higher fertilization rate at ICSI	11.4 ± 1.9	10.3 ± 1.5	0.108
Eftekhar et al. (49)	Yes	Poor responders; Iranian	40/42 (36.0 ± 4.6 y/36.2 ± 3.7 y)	previous failed IVF-ET cycles with ≤3 oocytes, and ≤3 embryos obtained; and/or E2 levels ≤500 pg/mL on hCG day	BMI ≥30, FSH >15 IU/L, endocrine or metabolic disorders, and PCOS, severe endometriosis and azoospermia	GnRH antagonist protocol; +treatment group 4 IU/d GH from day 21 from previous cycle until hCG triggering	Higher number of retrieved oocytes and obtained embryos, while no effect on implantation and pregnancy rates	8.5 ± 1.0	8.1 ± 0.9	0.158[a]
Bayoumi et al. (50)	Yes	Poor responders; Egyptian	72/73 (34.9 ± 4.9 y/34.8 ± 5.6 y)	ESHRE consensus criteria 2011 for poor responders	FSH >20 IU/l; previous ovarian surgery; infertility other than poor ovarian response; endocrine disorder; male factor infertility	GnRH agonist (microflare) protocol; +treatment group 7.5 IU/d GH from day 6 of hMG stimulation until day of hCG triggering	Higher number of mature oocytes and embryos obtained, while no effect on implantation and pregnancy rates	11.9 ± 1.6	11.7 ± 1.7	0.590[a]
Dakhly et al. (51)	Yes	Poor responders; Egyptian	74/74/68/71* (36.4 ± 5.8 y/38.1 ± 5.0 y/36.8 ± 6.3 y/36.4 ± 5.8 y) *Comparison of 4 different GH protocols, no control group	ESHRE consensus criteria 2011 for poor responders	>45 y; FSH >20 IU/l; previous ovarian surgery; other causes of infertility (other than poor responder); male factor of infertility	Gr1: GnRH long protocol; Gr2: GnRH short protocol; Gr3: GnRH antagonist protocol; Gr4: GnRH miniflare protocol. In all groups 7.5 IU/d GH from day 6 of hMG stimulation until day of hCG triggering	The long/GH (Gr1) protocol was superior regarding the number of oocytes retrieved and fertilized. No significant differences in pregnancy rates	11.5 ± 1.6 (Gr1); 11.4 ± 1.6 (Gr2) **12.1 ± 1.4 (Gr3); 11.1 ± 1.8 (Gr4)**	NA	**0.003**[a] (Gr3 vs. Gr4)
Bassiouny et al. (13)	Yes	Poor responders; Egyptian	68/73 (35.8 ± 5.6 y/35.5 ± 6.0 y)	ESHRE consensus criteria 2011 for poor responders	FSH >20 IU/l; previous ovarian surgery; infertility other than poor ovarian response	GnRH antagonist protocol; +treatment group 7.5 IU/d GH from day 6 of hMG stimulation until day of hCG triggering	Higher number of mature oocytes and embryos obtained, while no effect on pregnancy rates	12.1 ± 1.3	11.6 ± 1.6	**0.029**[a]

(Continued)

TABLE 1 | Continued

Study	RCT	Study group; Ethnicity	GH/control (mean age)	Inclusion criteria	Exclusion criteria	Intervention	Primary outcome	Effect on endometrial thickness (mm)		
								GH	Control	*p*-value
Du et al. (16)	No	Normal responders; Chinese	556/558 (32.8 ± 4.3 y/31.6 ± 4.4 y) (*__older women ≥35 y: 278/265__; **younger women <35 y: 278/293)	20-45 y; fallopian tube malfunction or male sterility; normal hormone levels; normal uterine cavity; regular menstrual cycles, BMI <25	Recurrent spontaneous abortion; severe pelvic adhesions or hydrosalpinx; cerebrovascular, liver or kidney disease; endocrine diseases; PCOS; endometriosis; uterine leiomyoma; adenomyosis	Long GnRH agonist protocol; +treatment group 4.5 IU/d GH for 5 days starting from day of FSH administration	Higher implantation and clinical pregnancy rates	12.2 ± 4.7 ***12.0 ± 2.2** **12.5 ± 7.0	11.8 ± 4.8 *11.6 ± 2.5 **12.0 ± 6.8	0.18[b] ***0.038**[b] **0.50 [b]
Choe et al. (52)	Yes	Infertile women with diminished ovarian reserve; Korean	62/65 (39.8 ± 3.6 y/39.4 ± 4.1 y)	≥40 y or any other factor for poor ovarian response; ≤3 oocytes with conventional stimulation protocol; antral follicle count <5–7 or AMH <0.5–1.1 ng/ml; normal uterus; regular menstrual cycle	Genetic cause for infertility; BMI >30; abnormal uterine bleeding; ovarian tumor; breast cancer; hydrosalpinx; contraindication for GH treatment	GnRH antagonist protocol; +treatment group sustained-release GH (20 mg) 3× before and during COS (mid-luteal, late luteal, cycle day 2)	Higher number of mature oocytes obtained, while no effect on pregnancy rates	8.8 ± 2.2	9.1 ± 1.9	0.24[a]
Dakhly et al. (53)	Yes	Poor responders; Egyptian	120/120 (36.4 ± 4.4 y/36.2 ± 4.5 y)	ESHRE consensus criteria 2011 for poor responders	>45 y; FSH >20 IU/l; previous ovarian surgery; other causes of infertility (other than poor responder); male factor of infertility	GnRH long protocol; +treatment group 7.5 IU/d GH from day 21 of previous cycle until day of hCG triggering	Higher number of oocytes and embryos obtained, while no effect on implantation and pregnancy rates	11.8 ± 1.3	11.3 ± 1.2	**<0.001**[a]
Chen et al. (19)	No	Recurrent implantation failure (RIF) patients; Chinese	22/20 (33.9 ± 2.9 y/34.0 ± 3.4 y)	Normal hormone levels; no use of synthetic hormones >3 months prior to entry	Prior endometrial resection or endometrial polyps; antiphospholipid syndrome; infectious disease; hyperthyroidism; hyperprolactinemia; chromosomal abnormalities; thalassemia; male factors	GnRH; +treatment group 4 IU/d GH through stimulation until the day of hCG administration	Higher clinical pregnancy and live birth rates	11.6 ± 2.9	9.7 ± 1.5	**0.009**[a]
Liu et al. (24)	No	Normal responders; Chinese	781/781 (31.3 ± 3.6 y/31.3 ± 3.3 y)	Normal ovarian response; age 20–40 y; poor quality embryos in previous IVF/ICSI; repetitive fresh or frozen ET without pregnancy	Poor or high ovarian response; adjuvant therapy as DHEA, CoQ10; serious and unstable diseases (cardiovascular, cerebrovascular diseases); recurrent spontaneous abortion; male factor infertility	GH treatment group 2 IU/4 IU GH daily since day 2 of previous cycle (6 weeks GH pretreatment) or day 2 from ovarian stimulation until hCG trigger (2 weeks GH pretreatment)	Increased pregnancy rate	12.0 ± 2.2	11.6 ± 2.8	**0.036**[a]

(Continued)

TABLE 1 | Continued

Study	RCT	Study group; Ethnicity	GH/control (mean age)	Inclusion criteria	Exclusion criteria	Intervention	Primary outcome	Effect on endometrial thickness (mm)		
								GH	Control	*p*-value
FROZEN EMBRYO TRANSFER/OOCYTE DONATION PROTOCOL										
Wu et al. (43)	NA	Patients with thin endometrium; Chinese	32/30 (NA)	NA	NA	HRT; +treatment group subcutaneous injections of GH	Improved endometrial blood flow and increased endometrial thickness	8.8 ± 1.3	7.1 ± 1.9	**<0.05**
Yu et al. (54)	No	Patients with persistent thin endometrium; Chinese	5/5* (32.2 ± 5.5 y) *same women served as controls before entering GH treatment	Regular menstrual cycle; use of artificial cycle; endometrium ≥7 mm; no abnormalities with hysteroscopy; <40 y; pelvic tubal or male factor infertility	NA	HRT; +GH treatment with 4–5 intrauterine GH perfusions of 6 IU GH diluted with 0.5 ml 0.9% saline on 9th to 12th day of the cycle (bed rest 15 min)	Improved endometrial thickness and receptivity	8.0 ± 0.6	5.8 ± 0.7	**<0.05**[b]
Xue-Mei et al. (23)	No	Infertile women undergoing FET; Chinese	77 Gr1/ 77 Gr2/ 76 controls (cycles; *n* = 240 women) (30.3 ± 4.1 y/31.3 ± 5.0 y/30.7 ± 4.3 y)	≤38 y; vitrified embryos not older than 2 y; ≥2 embryos frozen	Congenital or acquired uterine malformation; endometrial polyps; submucosal fibroids; intrauterine adhesion; severe endometriosis or adenomyosis; diabetes mellitus; abnormal blood clotting	HRT with oral estradiol valerate from cycle day 3. +treatment group 1 (Gr1): 4 IU/d GH injections from cycle day 8 until prog injection; +treatment group 2 (Gr2): 4 IU/d GH injections from cycle day 3 until prog injection	Higher implantation, clinical pregnancy and live birth rates	9.2 ± 0.9 (Gr1); **9.6 ± 1.0 (Gr2)**	9.2 ± 0.8	**<0.001**[b]
Altmäe et al. (20)	Yes	RIF patients with fresh donated oocytes; Spanish	35/70 (42.2 ± 4.5 y/42.4 ± 3.7 y/43.8 ± 2.5 y) (35 GH RIF; Control Gr1 35 nonGH RIF; Control Gr2 35 pos controls undergoing 1st oocyte donation)	RIF (≥2 implantation failures); 30–51 y	NA	GnRH agonist + oral estradiol; +treatment group daily injections of 1 mg GH (~3 IU) for 10 days of proliferative phase induced by exogenous oral estradiol. 1–2 days later vaginal P treatment was started	Higher implantation, pregnancy and live birth rates	9.3 ± 1.5	8.6 ± 1.0 (Gr1 non-GH); 9.4 ± 1.7 (Gr2 pos control)	**0.046**[b]
Yang et al. (22)	No	Patients with thin endometrium; Chinese	184/61 (cycles; *n* = 225 women) (33.7 ± 3.6 y/33.7 ± 3.4 y)	<40 y; receiving 2 blastocysts; endometrial thickness <8 mm on prog administration day. All patients with hysteroscopy for adhesions before FET	Uterine malformations; severe endometriosis or adenomyosis; tumor; diabetes mellitus; immune abnormalities	GnRH agonist + estradiol valerate from day 2–3 of cycle+ vaginal estradiol after menstruation + prog for 5 days; + treatment group 4.5 IU GH every alternate day subcutaneously injected from day of prog administration until ET	Higher clinical pregnancy and implantation rates	6.6 ± 2.9	6.7 ± 0.7	0.24[c]

(Continued)

TABLE 1 | Continued

Study	RCT	Study group; Ethnicity	GH/control (mean age)	Inclusion criteria	Exclusion criteria	Intervention	Primary outcome	Effect on endometrial thickness (mm)		
								GH	Control	*p*-value
Cui et al. (21)	Yes	Patients with thin endometrium; Chinese	40/53 (29.8 ± 3.0 y/29.7 ± 3.6 y)	Endometrium ≤7 mm; <40 y; normal ovarian reserve; fresh ET canceled due to thin endometrium; ≥2 D3 embryos frozen	Uterine anomaly; intrauterine adhesion; endometrial polyp; adenomyosis; malignancy	Oral estradiol valerate from day 3 of cycle until day 18 + virginal estradiol on days 15–18 of cycle. +treatment group 5 IU/d GH subcutaneous injections cycle days 15–18	Higher implantation and clinical pregnancy rates	7.9 ± 0.7	6.3 ± 0.9	**<0.001**[b]

[a]*Day of hCG administration.*

[b]*Day of ET.*

[c]*Day of progesterone administration.*

AMH, anti-Müllerian hormone; BMI, body mass index (kg/m^2); COS, controlled ovarian stimulation; ET, embryo transfer; FET, frozen embryo transfer; GH, growth hormone; HRT, hormone replacement therapy; GnRH, gonadotropin-releasing hormone; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; ICSI, intracytoplasmic sperm injection; IU, international unit; NA, not applicable; PCOS, polycystic ovarian syndrome; Pos, positive; Prog, progesterone; RIF, recurrent implantation failure; RCT, randomized clinical trial. Bold text highlights the GH group of significant difference from control group.

Thin Endometrium

Infertile women with thin endometrium represent another potential patient population that could benefit from the GH administration. All studies on GH co-treatment during treatment of infertile women with thin endometrium were conducted in frozen embryo transfer (FET) cycles, where GH was administered during the endometrial preparation for FET (21, 22, 43, 54) (**Table 1**). The largest study by Yang et al. was conducted on 225 infertile women, and did not detect any significant GH effect on endometrial thickness, while reporting significantly higher clinical pregnancy and implantation rates (22). They assessed GH effect on endometrial thickness on the day of progesterone administration, which could explain the difference in their results from the rest of the studies. The other three studies all noted significant improvement in endometrial thickness on the day of embryo transfer among patients with thin endometrium after administering GH throughout the FET cycle (21, 43, 54), and significantly higher implantation and clinical pregnancy rates (21). Wu et al. study also detected improved endometrial blood flow in the GH-administered patient group (43), similar to later findings by Xue-Mei et al. study (23), who showed increased VEGF expression and improved perfusion of the uterine arteries in the group of infertile women treated with GH. In line with above, Cui et al. study detected VEGF up-regulation together with ITGB3 and IGF-1 in endometrial cells when exposed to GH (21). The state of high blood flow resistance and VEGF down-regulation with inadequate epithelial growth and vascularization have been described as pathophysiologic characteristics of thin endometrium (62), and subendometrial blood flow on the day of embryo transfer is related to the implantation and pregnancy rate in IVF (63). Cui et al. concluded that up-regulated VEGF in their study setting, in the GH group, partly resulted in the increase of subendometrial blood flow and thereby improved endometrial receptivity (21). Nevertheless, the exact mechanisms of GH actions on the endometrium and endometrial receptivity in general are to be unraveled in future studies. Also new studies with larger study groups and well-designed RCTs are required in order to clarify whether infertile women with thin endometrium benefit from the GH treatment.

Poor Responders

Women with poor ovarian response in ART is another patient group where GH co-treatment in stimulation protocols have been studied. All these studies (see **Table 1**) have been RCTs, however with limited sample sizes, and all have reported beneficial effect of GH administration on the number and quality of oocytes and on the number of embryos obtained. Remarkably, while some improvement of endometrial thickness has been noted, those studies failed to show any beneficial effect on clinical pregnancy and live birth rates (13, 49–53). Based on these findings, one could conclude that GH co-treatment in poor responders with normal endometrium does not seem to have any significant impact on endometrial receptivity and hence pregnancy rates. Nevertheless, we should be cautious in drawing preliminary and potentially wrong conclusions in this type of studies without taking into careful consideration all potential confounders, including quality and number of embryos transferred, cleavage

vs. blastocyst stage embryos and even type of luteal support provided in fresh and/or frozen embryo transfer cycles (64). In addition, the total productivity rate from a single oocyte retrieval is highest when more and better quality embryos are obtained, which can be exactly the case with GH-supplemented cycles in poor responders, resulting in higher cumulative pregnancy rates rather than per cycle success in this group of patients. Clearly, carefully designed large studies with transfers of single good quality embryo (fresh and frozen) are warranted, albeit quite challenging to perform, in order to clarify whether endometrial receptivity in infertile women with poor response in ART would benefit from GH administration.

Normal Responders

Thus far, the largest group of infertile patients involved in studies on GH administration during IVF has been the normal responders (**Table 1**). The first study was performed on 240 infertile women undergoing FET, where two different GH supplementation protocols were compared—GH administration throughout the FET, and a single GH injection on day 8 of estrogen treatment (23). Notably, significant endometrial thickness improvement together with higher embryo implantation, clinical pregnancy, and live birth rates were detected among women with longer GH administration (23). The authors also noted that the longer GH addition to the treatment protocol increased the levels of estradiol, IGF-1, and VEGF serum levels, and improved perfusion of the uterine endometrial arcuate artery (23). The pulsatility index, resistance index, and peak systolic velocity/end diastolic velocity of the uterine arcuate arteries represent the resistance of blood flow from the point of measurement downstream; increased impedance of these arteries might correlate with poor endometrial receptivity and clinical outcomes (65).

The next studies analyzed 1,114 (16) and 1,562 (24) infertile women, respectively undergoing ovarian stimulation for IVF with GH co-administration throughout the stimulation, and a positive GH effect on endometrial thickness in addition to the higher clinical pregnancy rates was detected in study compared to control groups. GH effect on endometrial thickness was significantly increased among older infertile women of $\geq$35 years old compared to <35 years old, while both groups exhibited higher implantation and clinical pregnancy rates, most likely attributed to the higher number of high quality embryos obtained in GH-treated groups (16). In humans, changes in GH secretion could be age-related, as post-adolescence the secretion of GH decreases with age, which is why GH hyposecretion is observed in older patients (66). GH insufficiency can disrupt ovarian function and lead to reproductive difficulties (66). As mentioned above, in Du et al. study (16), GH-treated older women ($\geq$35 years old) had implantation and clinical pregnancy rates more than two times higher than those observed during IVF cycles without GH. This result suggested that adding GH might be beneficial for older patients.

To conclude, research on the effects of GH co-treatment in ART among normal responders has been performed on sufficiently powered studies in terms of the sample size, nevertheless as all these studies were not randomized controlled trials, further well-designed research is needed to objectively assess the GH effect on ART outcomes in (young) women with normal ovarian reserve and normal response to ovarian stimulation.

Future Perspectives

Further studies are warranted in order to determine the optimal dose, time, and duration of GH administration and to investigate the long-term safety of GH for patients and their offspring. The dosage and treatment duration of GH differed among conducted studies (see **Table 1**). Because of the limited experience with the GH co-treatment protocols, there is a lack of evidence to support the superiority of one over the other. In all the protocols used (see **Table 1**), GH was administered via subcutaneous injections, except for one study where GH intrauterine perfusion in 5 patients with non-responsive thin endometrium was successfully used (54).

Another crucial part is to define the appropriate patient population that would truly benefit from GH treatment for improving their uterine lining quality in terms of thickness and/or receptivity. GH seems to promote endometrial growth, and its use could be considered in women whose endometrium does not grow and/or mature sufficiently with standard treatment protocols. In addition, the current review concludes that even normal responders could potentially benefit from the GH administration in IVF programs, however, the improved pregnancy rates in some of the studies utilizing fresh IVF cycles could not be separated from improved embryo quality. While endometrial thickness and pattern upon GH administration has been recorded and reported, evaluation of endometrial receptivity is not as simple. Future studies need to focus on the molecular level in order to evaluate the endometrial transcriptome/proteome/secretome (67), with emphasis on receptivity markers to understand and clarify the possible mechanisms of GH on endometrial receptivity. An ideal setting would be to design an RCT with GH-supplemented mock cycles vs. control, during which endometrial receptivity could be studied on molecular level in detail (transcriptomics and/or use of commercially available endometrial receptivity tests; epigenomics and/or proteomics analyses). The mock cycle could be followed by a "true" FET cycle to enable evaluation and correlation to pregnancy rates. To sum up, undoubtedly more research on larger cohorts with carefully designed studies [as highlighted in a recent comment (64)] is needed to identify the patient group in whom the addition of GH to the treatment protocol in IVF programs will be most valuable.

Sample size and objectively designed studies (randomized clinical trials) is a delicate topic in ART as strict double-blind, placebo-controlled, RCTs are difficult to accomplish (68). It is extremely hard to perform fully blinded RCTs in IVF because of the patient recruitment issues, where aging women prefer not to participate in the placebo group that requires commitment for several months of their reproductive lifespan and which ultimately may not help them achieve pregnancy (68). Understandingly, patients tend to opt for any additional treatment, cost permitting, that would potentially help them to become pregnant. As a result, the studies of GH treatment effects

on IVF outcomes are rather limited on its sample size and/or are retrospective or observational in nature; nonetheless, they provide important data concerning therapeutic interventions in IVF and open up future possibilities for improving infertility treatment protocols.

CONCLUSIONS

The current review summarizes the recent data on GH co-treatment effects on endometrial parameters in assisted reproduction and proposes possible mechanisms of GH actions in the endometrium. Studies are indicating that co-treatment with GH could improve the endometrial thickness, and possibly receptivity among infertile women. This effect might occur through increasing endometrial blood perfusion and the expression of genes and proteins related to endometrial receptivity such as VEGF and ITGB3 together with IGF-1, however the exact mechanisms in the endometrium remain to be clarified.

Whether GH administration during IVF is useful and which patient groups could benefit from it needs further investigation, but the preliminary data suggest that women suffering RIF, patients with thin endometrium and older normo-responders could benefit from GH treatment when undergoing ART. Still, carefully designed and sufficiently powered cohort studies, RCTs, are required in the field in order to establish the most suitable therapeutic regimen for these patients and to clarify the confusion arisen from various studies that have shown either inconsistent or conflicting findings, used small patient cohorts and/or have been poorly designed with no blinding or placebo controls.

AUTHOR CONTRIBUTIONS

SA and LA equally contributed to the review idea and manuscript writing.

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REVIEW
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The Concept of Growth Hormone Deficiency Affecting Clinical Prognosis in IVF

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The current understanding of human growth hormone (hGH; here GH) action is that the molecule is a 191-amino acid, single-chain polypeptide that is synthesized, stored and secreted by the somatotroph cells within the lateral wings of the anterior pituitary gland. It can be classified as a protein (comprising more than 50 amino acids) but true proteins have tertiary and quaternary chains creating a more complex structure, hence GH is usually classified as a polypeptide. GH is normally secreted at night during sleep and promotes skeletal, visceral and general body growth through the action of somatomedins or IGFs, notably IGF-1. In some tissues, GH action is directed via specific receptors GHRs; these are most abundant in liver, adipose and muscle tissues but have also been shown in granulosa cells, testicular tissues and on the oocyte, as well as in glandular cells of the luteal phase endometrium and decidua; such findings being recent and minimally researched to now. Following engagement with its receptor, the transduction process activates multiple signaling proteins. These all lead to extensive metabolic and mitogenic (growth promoting) responses. Clinically, GH is known to have an important role in pubertal development and is a key hormone for the vigor associated with adolescence and early adult life stages but has a faded presence and role for later adulthood, beyond age 30 years, and is minimally detected in advanced age, beyond 40 years. In association with the rapidly increasing trend for delaying reproduction beyond age 35 years, GH is being widely researched now as a potential adjuvant for infertility treatment in this group who, studies consistently show, have a poorer prognosis than younger females when relying on autologous oocytes. The idea that the age-related reduction in fertility prognosis is a feature of growth hormone deficiency is supported by our studies showing an elevated binding protein IGFBP-3/IGF-1 ratio and this can be reduced to a normal range (matching younger, good prognosis women) by the administration of GH as an adjuvant.

Keywords: growth hormone GH, growth hormone receptor GHR, follicle stimulating hormone receptor FSHR, bone morphogenetic protein receptor BMPR, luteinising hormone receptor LHR, insulin-like growth factor-I IGF-I, growth hormone deficiency GHD, adult growth hormone deficiency AGHD

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INTRODUCTION

This article explores the physiological processes which might support the clinical findings which indicate a benefit for growth hormone (GH) as an adjuvant in the treatment of women who fail to conceive from *in vitro* fertilization (IVF) treatments. Such women may be categorized as "poor-prognosis" due to a range of categories including poor ovarian responses (POR) to high-dose

gonadotrophin stimulation; advanced female age (≥40 years); low ovarian follicle reserve defined by a low antral follicle count (AFC) or low serum level of anti-mullerian hormone (AMH); the failure to generate good-quality embryos after fertilization of their oocytes; or simply the failure to gain successful implantation with resultant pregnancy and livebirth outcomes, so called recurrent implantation failure (RIF). A large number of adjuvants have been explored in the attempt to improve the prognosis for such women, but none of the studies have reached the desired high standard expected from evidence-based medicine (EBM) which requires a number of RCTs to reach meta-analysis support for the particular adjuvant. Where RCTs have been attempted, they have fallen short on recruitment processes and inadequate numbers. This current clinical story has been fully covered in a specific article from our group in this e-book (Yovich et al., under review) which traces the evolution of the "poor-prognosis" concept and indicates that the observational and retrospective studies for GH are strongly supportive of this adjuvant over others which have been reported. Given the recent recognition of the limitations of RCT application in the area of adjuvants or "add-ons" (1), we believe our data reports on GH may be the best achievable currently.

The notion of using growth hormone (GH) as an adjuvant for women in need of assisted reproduction dates back to observations in 1969 followed by studies reported over the 25-year period from 1972 to 1995.

HISTORICAL STUDIES

One of the earliest to consider the idea was Howard Jacobs, a London-based endocrinologist whose special interest was disorders of ovulation, particularly in association with polycystic ovary syndrome and metabolic disturbance. It was 1969 when Jacobs showed that patients with primary or secondary impairment of adrenal cortical function responded poorly to a wide range of illnesses, injuries or surgical operations, especially those with poorly controlled diabetes (2). This association was determined by measuring 11-hydroxycorticosteroid levels which are very high in appropriate responders but low in those patients who are failing to recover. Concomitant measurements of GH (by a radio-immunoassay sensitive to 0.4 mug/ml) revealed that GH levels are similarly elevated in uncontrolled diabetes, normalizing as insulin response reduces plasma glucose and improves intracellular glucose economy. Jacobs surmised that the hypothalamic-pituitary-adrenal response could somehow also influence GH output in response to various physiological insults, although he was reluctant to draw strong conclusions as GH levels tended to vary widely, even in recovered patients.

Thereafter, the Jacobs' team explored the use of GH in women with amenorrhoea who had shown resistance to ovarian stimulation using human menopausal gonadotrophin (HMG) stimulation (3). The women all had hypopituitarism from a range of causes, many post-surgical from various pituitary tumors, some Kallman's syndrome, others unexplained, and a few with underlying polycystic ovaries. In blinded placebo-RCTs, Jacobs and his team showed that the dosage of HMG required to induce ovulation was significantly reduced (by 30%) and the duration of stimulation was also significantly reduced (by 5 days); when GH was given as an adjuvant. The Insulin-like growth factor-1 (IGF-I) levels rose significantly (by double) but IGF-II levels did not change. These and further findings from Jacobs' team (4–6) "confirmed that GH sensitizes the human ovary to the stimulatory effects of treatment with gonadotrophins." In a large multi-center study involving several centers from the United Kingdom along with centers in Australia and Sweden, Jacob's group conducted a prospective randomized, placebo-controlled, dose-response study evaluating GH co-treatment with gonadotropins for ovulation induction in hypogonadotropic patients. The findings confirmed the previous studies and showed that GH had an amplification effect of gonadotropin on the ovary and thereby reduced the gonadotropin dosage required to induce ovulation (7). However, whether the effect of GH was exerted directly on the ovary or via the IGF-I system was left unanswered at that time.

Workers from other locations added further useful knowledge. At Stanford University, USA the team of Aaron Hsueh had extensively researched factors influencing growth and organ function, particularly the influence of hormones and growth factors. They also specifically examined the possible direct effect of GH on the differentiation of granulosa cells from the ovaries of hypophysectomised estrogen-treated rats, reporting several studies across the period 1983–1986 (8–10). These *in-vitro* studies revealed that follicle stimulating hormone (FSH) stimulated luteinizing hormone (LH) receptor formation and steroid production in a dose-dependent manner. Concomitant treatment with GH increased LH receptor content by enhancing the action of low doses of FSH. Their data demonstrated that GH augments gonadotropin-stimulated differentiation of ovarian granulosa cells, suggesting an important regulatory role of GH in follicular growth as well as in pubertal development. From similar rat studies in Melbourne, Australia in 1987, the research team of Jock Findlay showed that both GH and IGF-I could independently enhance aromatase activity induced by pregnant mare serum gonadotrophin (PMSG) with elevated estrogen as well as progesterone production; and the stimulatory actions would continue after the gonadotrophin was removed from the culture medium (11, 12). Thus, both GH and IGF-I act on FSH-induced granulosa cells to accelerate the differentiation of the follicular cell to a lutein cell and this was mostly independent of cyclic adenosine monophosphate (cAMP). Findlay's team extended their studies to bovine, sheep, pigs and chicken and showed that a range of growth factors, derived from thecal cells, including epidermal growth factor (EGF) and fibroblast growth factor (FGF) influence not only proliferation but functional differentiation of ovarian follicle cells. Two others, namely transforming growth factor-type ß (TGF-ß) and platelet derived growth factor (PDGF), modulate these actions, sometimes directly opposing them to suggest an inverse relationship between differentiation and mitosis. By 1995, Findlay concluded that there was sufficient evidence supporting the ability of GH to influence ovarian function and proposed that GH was a co-gonadotrophin that synergises with FSH and LH in the promotion of ovarian function. Resolving

the unanswered question of Jacobs in 1988, regarding the mechanism (3), he showed this could be manifest in two ways, not necessarily mutually exclusive. On the one hand GH could act via its receptors, resulting in direct modulation of the action of gonadotrophins on ovarian somatic cells. This implied an interaction between the second-messenger systems within the target cell subserving each of the pituitary hormones. On the other hand, GH could act via its receptors to stimulate the production of IGF-I that in turn could have autocrine or paracrine actions on the ovarian somatic cells to modify the actions of FSH and LH. Implicit in this second possibility is the presumption that the ovarian expression of the IGF-I gene and the intra-ovarian actions of IGF-I are either partially or totally GH dependent (13).

Another team from the USA, assembled by Eli Adashi in Maryland, explored growth factor involvement in ovarian maturation with many studies on rat granulosa cells reported across the period 1984–1988 (14, 15). In essence Adashi showed that IGF-I amplified FSH action, consistent with the aforementioned studies. Adashi had in the early 1980's undertaken granulosa cell studies with Hsueh, whom he gratefully acknowledged as one of his mentors (8, 9).

CLINICAL ASPECTS OF GROWTH HORMONE DEFICIENCY

Classically, the diagnosis of growth hormone deficiency (GHD) has been confined to pre-pubertal children. The clinical picture has a wide range of manifestations including growth failure, particularly height persistently falling below the fifth centile and children are treated by GH injections through to puberty. Thereafter it had been considered that there is no further need to maintain GH supplementation. However, a number of reports in recent times have shown that adults who had been treated for GHD in childhood had definable conditions in their adult years, particularly related to obesity and diminished cardiac function. For example, a recent Israeli report documenting the post puberty development of 39 persons (23 males and 16 females) with childhood GHD who ceased GH at puberty, exhibited delayed further growth and a progressively increasing development of obesity in their adult years (16). Twelve of them suffered from hyperlipidemia, four developed diabetes mellitus, and five developed serious cardiovascular diseases. One patient died in an accident. None developed cancer. Of the 39 patients, 22 have an education level of high school or higher, and 2 are in special institutions. Most are employed in manual labor. It was concluded that patients with childhood GHD who do not receive early and regular replacement treatment are prone to lag in achieving normal height and suffer from educational and vocational handicaps.

In 2011 The Endocrine Society has issued a report following evaluation of systematic reviews and is now accepting (conceding) the diagnosis of adult growth hormone deficiency (AGHD). That report concludes that *"GHD can persist from childhood or be newly acquired. Confirmation through stimulation testing is usually required unless there is a proven genetic/structural lesion persistent from childhood. GH therapy offers benefits in body composition, exercise capacity, skeletal integrity, and quality of life measures and is most likely to benefit those patients who have more severe GHD. The risks associated with GH treatment are low. GH dosing regimens should be individualized. The final decision to treat adults with GHD requires thoughtful clinical judgment with a careful evaluation of the benefits and risks specific to the individual"* (17).

The Endocrine Society had previously been reluctant to entertain the notion of AGHD as the symptoms are wide-ranging, non-specific and may reflect the natural aging process. The society has been careful not to feed into the idea of widespread use of GH to defer or allay the natural age-related decline in muscle strength and exercise tolerance. Diagnosing newly acquired GHD requires specific testing which can include the insulin tolerance test (ITT) and the growth hormone releasing hormone (GHRH)—arginine stimulation test. These are best undertaken by an endocrinologist as interpretation of the findings can sometimes be complex, although associated low IGF-1 levels tend to clarify the clinical picture. In those adults who had previously been diagnosed as GHD in childhood, low IGF-1 levels alone may be accepted as diagnostic and indicative of the need to re-establish GH therapy. The Endocrine Society also considers the presence of deficiencies in three or more pituitary axes along with low IGF-1 levels, is also sufficient to make the diagnosis without resorting to stimulation testing. This means hypothyroidism, hypogonadism (testosterone or oestradiol deficiency), hypoadrenalism and/or hypo-prolactinaemia combined with low IGF-1 is sufficiently diagnostic to warrant GH therapy.

More recently the NICE Guidelines (18) state that GH therapy for the treatment of adults is recommended only if they fulfill all three of the following criteria:

1. GH deficiency is demonstrated, defined as a peak GH response of <9 mU/l (3 ng/ml) during an ITT or a cross-validated GH threshold in an equivalent test.
2. They have perceived impairment of quality of life (QoL) as demonstrated by a reported score of at least 11 in the disease-specific QoL assessment of AGHD questionnaire.
3. They are already receiving treatment for other pituitary deficiency disorders.

NICE recommends a 3-month period for dosage titration of GH, thereafter a 6-month trial of GH therapy. At 9 months the QoL assessment questionaire should be reviewed with a view to ceasing GH therapy if the score fails to increase by at least seven points. For those who had GH in childhood, the use of GH in adult life is predicated on achieveing peak bone mass, thereafter ceasing unless QoL parameters are reduced.

Furthermore, NICE recommends that the "*Initiation of GH treatment, dose titration and assessment of response during trial periods should be undertaken by a consultant endocrinologist with a special interest in the management of GH disorders. Ongoing treatment should be conducted in a shared-care arrangement with the Endocrinologist as the lead clinician*".

The above advice should be considered in the context that NICE technology appraisal guidance is about the use of new and existing medicines and treatments in the NHS in England and Wales. In other jurisdictions, the logistics and funding

requirements may be quite different leading to compromise in managing AGHD.

Approximately 20–25% of women attending IVF clinics may be categorized as poor-prognosis and be considered for adjuvant therapy, often at the woman's own request. In the consideration of the aforementioned NICE and Endocrine Society guidelines, it may appear that few of these women could fulfill the clinical criteria advised for GH therapy. However, in the context of changing features among the infertile population, we clinicians may need to probe our patients more deeply. Although they may present as ostensibly healthy, the profile of poor responders reflects an older population, prone to higher BMI levels and have subclinical metabolic syndrome (19). If appropriate investigations are performed (with careful cardiovascular assessment, along with lipid profile, GTT and the consideration of ITT where indicated), we may take heed of the first, 1969 citation from Jacobs in this review article; where he demonstrated that reduced GH levels are associated with poor recovery from a range of serious illnesses. Favorable definitive evidence of clinical benefit from GH treatment in AGHD cases is only now beginning to emerge. A recent report involving improvements to specific cardiac prognostic parameters (20) is greeted with cautious optimism. Perhaps women requiring assisted reproduction and are classified as poor prognosis, can be considered to have a subclinical degree of AGHD.

WHAT CONSTITUTES A LOW IGF-1 LEVEL?

Recent reviews concerning GH and IGF-1 show a complex inter-relationship, differing with respect to the natural, pituitary secretion of GH vs. exogenous GH; or hepatic vs. intragonadal (ovary or testis) secretion of IGF-1 (21). The emerging theory is that IGF-1 is an autocrine growth stimulator of follicles and plays a key role at different stages of follicular development. Whilst it appears that IGF-1 is not required for primordial to primary follicle transition, it is necessary for the development of small antral follicles at the gonadotropin-dependent stages (22). Furthermore, IGF-1 increases granulosa cell proliferation, steroidogenesis and oocyte growth (23, 24). It also appears that follicular fluid IGF-1 is a biochemical marker of oocyte quality, providing predictive power of embryo quality and subsequent implantation rates in IVF (25).

Most publications examining IGF-1 ranges report in traditional units (ng/ml) with normal ranges ∼180–400 ng/ml but these do have to be adjusted for age. A recent study reporting on healthy Chinese adults shows that gender and age both influence IGF-I levels and there is a gradual decline in levels with advancing age in all adults (26). For example, at age 20 years the median level of IGF-1 is 280 ± 60 ng/ml in males and 300 ± 60 ng/ml in females. At 35 years, the median level is 200 ± 50 ng/ml in males and 220 ± 60 ng/ml in females. At age 45 years, the corresponding levels were 180 ± 40 ng/ml and 200 ± 40 ng/ml, respectively. The matching SI units applies a conversion factor of 76.5 ng/ml equating to 10 nmol/L with a normal range of 200 ng/ml equating to 26.1 nmol/L in males and 20.9 nmol/L for females at the lower standard deviation (SD) point. In practical terms for women aged between 30 and 40 years, the 5th centile (2SDs) equates to 20 nmol/L. However, IGF-1 is known to be carried on six binding proteins, the main one being IGFBP3. In young adults the levels range from 120 to 180 nmol/L and tend to be very stable in individuals. Hence it has been proposed that a ratio may provide the clearest picture regarding IGF bioavailability. This can be reported as IGF-1/IGFBP3 when the ratio for healthy young adults will range 0.15–0.4 (27–30). This can be placed in reverse with IGFBP3/IGF-1 ratio ideally at 3.0 (e.g., 120 nmol/L divided by 40 nmol/L). A ratio <1.6 correlates with an acromegalic state and ratios >4.4 are consistent with GHD. In our own (PIVET, yet unpublished) studies on subfertile women we have regarded IGF-1 levels <20 nmol/L as representing the deficiency range and an IGFBP3/IGF-1 ratio ≥ 5.0 being consistent with AGHD, implying that such women will be likely to benefit from GH supplementation.

WHY NOT SIMPLY MEASURE GH?

Human GH (hGH, here GH) is a 191-amino acid, single-chain polypeptide hormone that is synthesized, stored, and secreted by the somatotroph cells within the lateral wings of the anterior pituitary gland. Because of its size (comprising more than 50 amino acids), GH could be termed a protein, but it is essentially a linear single-chain polypeptide without the complex foldings with tertiary and quaternary chains which typify true proteins. GH action is directed via specific receptors GHRs; these are most abundant in liver, adipose and muscle tissues but have also been shown in granulosa cells, testicular tissues and on the oocyte, as well as in glandular cells of the luteal phase endometrium and decidua; such findings being recent and minimally researched to now (20). The transduction process for GH is via the Janus kinase signal transduction and activation of transcription (JAK-STAT) signaling pathway after induction of GHR dimers which then activate two JAK2 molecules. This, in turn results in phosphorylation on multiple GHR tyrosines, in turn activating multiple signaling proteins including STATs 5A and 5B, insulin receptor substrate IRS, phosphoinositide PI-3 kinase, extracellular signal-related kinase ERK or mitogen activated protein kinase MAPK. The serine/threonine specific kinase B (PKB, also designated Akt) is also involved in the resulting protein synthesis and inhibition of apoptotic processes. These all lead to extensive metabolic and mitogenic (growth promoting) responses. Pituitary-derived GH, the main serum source, is normally secreted in a 90-min pulsatile fashion, mostly at night during sleep and activates cell-surface receptors directly. However, locally produced GH is continuously generated and activates receptors on the endoplasmic reticulum. These somatotrophins both promote skeletal, visceral and general body growth through the action of somatomedins or insulin-like growth factors although the pattern varies. In particular GH raises serum IGF-1 and IGF-II levels and these proteins are also known as Somatomedin C and A, respectively. They are both growth-promoting proteins with IGF-II mainly active during fetal gestation and IGF-I during adult life. Both

somatotrophin (GH) and somatomedins (IGF's) have a variety of effects on lipid, protein and carbohydrate metabolism. The somatomedins stimulate somatostatin from the hypothalamus which suppresses GH release and this creates a negative feedback mechanism on both GH as well as on their own production. Although the liver is the main source of circulating IGF's, the somatomedins are also produced within many tissues where they have both autocrine and paracrine actions in addition to their endocrine action. The current immunoassays have improved the methodology over previous radio-immunoassays, but small peptide interference continues to affect their reliability. So too, does the pulsatile nature of GH release create extremely wide variability in GH detection, such that zero levels can still be consistent with clinical normality. On the other hand, the IGF-I assays have improved with very low coefficients of variation over a wide range lending clinical consistency and reliability (26).

Clinically, GH is also known to have an important role in pubertal development and is a key hormone for the vigor associated with adolescence and early adult life stages for both males and females, but has a faded presence and role for later adulthood, beyond age 30 years, and is minimally detected in advanced age, beyond 40 years. This pattern coincides with the current challenge of managing infertility where female age is the overwhelming limiting factor and future strategies include oocyte preservation at young age, strategies to improve oocyte quality *in-vitro* and stem cell transformations (31). So far, the idea of GH therapy as a specific treatment for older subfertile women has yet to be suggested, let alone studied in an appropriately designed research trial.

IGF-I LEVELS IN ASSISTED REPRODUCTION

So far there are few reports covering the diagnosis of AGHD in assisted reproduction, and those being only case reports or observational on small case numbers (32). At PIVET, we have included IGF-1 and IGFBP3 testing on all new consultations over the past 5-years as part of their formal Assessment Cycle. Furthermore, cases provided with GH as adjuvant treatment on the basis of poor prognosis categorization have their levels and ratios reviewed 4-weeks after commencement of GH. Such data will be presented as a retrospective analysis. In the meantime, we have some earlier pilot data from 190 women attending for IVF (presented, but not published) which encouraged our current studies. These showed that, across all age categories (<35, 35–39, ≥40 years), IGF-I levels ranged from 9 to 52 nmol/L with a mean of 24.4 nmol/L. Whilst the younger women (<35 years had a higher mean level (25.7 nmol/L) than women ≥35 years (23.1 nmol/L) the ranges were equally wide. The IGFBP3 levels ranged 101–237 nmol/L with an overall mean of 162.5 nmol/L and tending to be higher in the women ≥35 years. Calculation of the IGFBP3/IGF-l ratios showed levels ranging from 3.3 to 13.8 with a mean of 7.2 (well above our cut-off limit of 5.0; implying AGHD affecting the majority of infertility cases. The mean of 6.7 for women <35 years was less marked than the ratio of 7.6 for those ≥35 years. Of greater interest was the finding of a marked improvement in IGF-I levels for 20 women treated with GH, rising from a mean level of 20 up to 34 nmol/L and which corresponded with a reduction of ratios from a mean high of 8.9 to a mean normal of 4.1. Although this data has not yet been tested by publication, it provides support for our continuing studies on GH as an adjuvant in IVF. In this respect it was reported by the Jacobs team in 1995 (7) that IGF-1 levels rose according to the dosage of GH applied. In their placebo-controlled study GH was administered by intramuscular injection alternate days over the course of gonadotropins to a maximum 7 injections (total dosage ranging 28 IU to 144 IU; as the higher dosages were not required beyond five or six injections). The 4 IU GH dosage caused an incremental rise of IGF-I by a mean of 10 nmol/l; 12 IU GH caused a rise of 20 nmol/L and 24 IU caused IGF-1 levels to rise by a mean of almost 30 nmol/L. Jacobs concluding remark in his 1995 report is pertinent stating "although the actual therapeutic role of GH in ovulation induction is at present unclear, the reality of its interaction with gonadotropins in now unequivocally established."

RECEPTOR STUDIES INVOLVING GH, FSH, LH, AND BMP

The first report of GH receptor (GHR) expression in the ovaries came from Israel in 2008 following studies on terminated fetuses as well as from girls and women requiring gynecological procedures (33). The proteins and mRNA transcripts for GH and GHR were detected in oocytes, granulosa cells and stroma cells from both sources (fetuses and women/girls), albeit with low staining intensity only in a portion of the fetal granulosa cells. This supported the earlier studies of GH involvement in ovarian function.

Co-author Sheena Regan has focused her studies on hormonal receptors in the ovary studying both sheep (the highly fecund Booroola sheep which carries a BMP mutation) (34) and human (focusing on women classified as poor-prognosis) (35–37). These human studies demonstrated dysregulation of the granulosa cell density of BMP 1B receptor as well as FSH and LH receptor density in women with reduced ovarian reserve and age-related infertility. This, in turn, adversely influences granulosa cell apoptosis. Her most definitive work shows that GH co-treatment increased the receptor density for FSHR, BMPR1B, LHR, and GHR in granulosa cells compared with the non-GH-treated patients of the same age and ovarian reserve (38). Furthermore, GH restored the preovulatory down-regulation of FSHR, BMPR1B, and LHR density of the largest follicles which may consequently improve the maturation process of luteinization in older patients who have reduced ovarian reserve. The fertility of the GH-treated patients improved accordingly with a significant increase in pregnancy rate.

UNDERSTANDING APOPTOSIS IN PERI-OVULATORY FOLLICLES

The aforementioned studies from the PIVET-Curtin collaboration has led to a changed view regarding depletion

of the ovarian reserve of primordial follicles and increased apoptosis of granulosa cells being related to poor quality oocytes in older women. On the contrary, apoptosis within the granulosa cells is an integral part of normal development and has limited predictive capability regarding oocyte quality or the ensuing pregnancy rate in IVF programs (39). In flow cytometry studies on the granulosa cells from the follicles of younger women undertaking IVF, the level of apoptosis was shown to be inversely related to the density of BMPRs as well as FSHR density. Conversely it was shown that this normal relationship became dysregulated. In the older patients the reduced apoptosis noted in the granulosa cells from the aspirated follicles at IVF (37) reflects the poor mitogenic growth turnover rate of healthy follicles rather than the death rate in an atretic follicle. It was proposed that restoring an optimum receptor density and down-regulation of receptors may improve oocyte quality (competence) with an improved pregnancy rate in older women. In fact, this has now been demonstrated in further studies on both apoptosis and the beneficial GH effects on FSH, LH, and BMP as well as GH receptors (38).

IMPROVING OOCYTE COMPETENCE BY GH FOR POOR PROGNOSIS CASES IN IVF

Further studies on GH adjuvant from our PIVET-Curtin collaboration have been published. One involved the detection of improved functional capacity of mitochondria in the oocytes of older women (≥35 years) treated with GH compared with an untreated group matched by age and poor-prognosis categories), as well as a young, good prognosis group (40). This study utilized immunofluorescent localization of GH receptors (GHRs) on the human oocyte and unbiased computer-based quantification of fluorescence following combined staining with mitotracker red for cellular viability, and cytochrome c oxidase for mitochondrial function. This enabled comparative assessment of oocyte quality between women of varying ages, with or without GH treatment. In this study we demonstrated for the first time, the unequivocal presence of GHRs on the human oocyte. Furthermore, the oocytes retrieved from the older women (classified as poor-prognosis) showed a significant decrease in the expression of GHRs and amount of functional mitochondria when compared with those from younger patients. Of further interest, when the older patients were treated with GH, a significant increase in functional mitochondria was observed in their oocytes. We concluded that GH exerts a direct mode of action, enabling the improvement of oocyte competence. This was achieved via the upregulation of its own receptors and enhancement of mitochondrial activity and may explain the clinical benefits from GH which we have separately reported (41, 42).

RECENT REPORTS DEMONSTRATING IMPROVED OOCYTE COMPETENCE FROM GH ADJUVANT

Accordingly, five other very recent clinical studies of GH use in IVF are of interest, beginning with a registered randomized controlled trial (RCT) from Cairo (43) where GH was added to the gonadotrophin stimulation phase of long-down regulation cycles applied in women classified as poor responders. Matching our own GH studies, the Cairo group demonstrated that significantly more usable embryos were generated under the influence of GH adjuvant. However, this did not translate into more infants, probably because of several procedural problems in their protocols as pointed out in a critical response (44), published in the same journal.

A second study from China describe the use of GH adjuvant in IVF cases categorized as RIF (45). This was an observational study where the treatment group of 22 women receiving GH injections were matched against 20 untreated cases. The GH group had both a higher pregnancy rate and live birth rate ($p < 0.05$) but this clinical aspect can easily be critically discounted on the grounds of an inadequate protocol and study design as well as low numbers. However, what was particularly interesting was the finding of elevated expression of hormone receptor (GHR) mRNA in the granulosa cells of the GH-treated group than the control group ($P < 0.05$) and the finding was positively correlated with GH levels in the follicular fluid ($r = 0.460$, $P < 0.05$). This indicated that GH adjuvant generated GHR responses which was likely to have underlined the favorable clinical responses.

A third study, again from a different province of China, compared clinical outcomes applying GH adjuvant for poor responders utilizing a mild stimulation protocol (46). The study had major design weaknesses being retrospective, the groups were not randomized, and the numbers (61 in the GH arm and 71 in the "control") were not sufficient to determine a significant clinical improvement; requiring 200 in each arm There was however a relevant finding of significantly higher numbers of good quality "usable" embryos in the GH group ($P < 0.01$). This finding matches the study reported from Cairo (43) which was also criticized for similar reasons (44).

The most recent, fourth study, this one a prospective RCT from Iran (47), showed GH-related improvements in clinical outcomes for women classified with POR. There were 3 arms in the GnRH antagonist regimen—one ($n = 34$) utilizing GH from day 3 of the previous cycle (∼20 days); a second ($n = 32$) utilizing GH from Day 8 of the gonadotrophin phase (∼5 days); and a third ($n = 28$) using a GH placebo (saline injections) from Day 8 of the gonadotrophin phase. The study described significantly lower pregnancy and live birth rates from the placebo arm, and equivalent good rates from both GH adjuvant arms (20 and 5 days of GH). Whilst these favorable outcomes can be heavily discounted because of the low recruitment numbers (the GH numbers should be ∼200 women and the placebo should also be ∼200 women) the embryology data can be accepted as the number of collected oocytes, MII oocytes, fertilized oocytes and embryo utilization rates were all highly significantly better in the GH groups (all $P < 0.001$). This is entirely in accord with the findings reported from our center (41, 42).

A fifth study, which is now in press, examines the outcomes of GH-generated embryos which have been cryopreserved by vitrification (48). From a total 2,857 frozen embryo transfer (FET) cycles, 1,119 women had GH-generated embryos transferred. Computerized case-matching enabled 3 similar

groups to be statistically analyzed for comparison, all single embryo transfers (SET) from autologous embryos—normal responders ($n = 809$) vs. poor prognosis; no GH ($n = 201$) and GH-derived embryos ($n = 109$). The pregnancy rates and live birth rates were significantly higher in the poor prognosis group where the embryos were GH-derived ($P < 0.005$ for both pregnancies and livebirths). Furthermore, tightly matched comparisons for age of the woman at FET ($n = 89$ in each group) and age of the woman at time of embryo generation ($n = 85$ in each group) showed that the GH-generated embryos had the same chance of implantation (equivalent pregnancy, live births and miscarriage rates) between the normal, good prognosis women) vs. the GH-generated poor prognosis women. This data further supports the idea that GH improves some aspect of oocyte quality which confers improved competency for implantation, and which is not detectable at morphological embryo grading.

ADVERSE CLINICAL EFFECTS OF GH

With its known effects on growth and metabolism, it was expected that patients on GH would be at risk of sequelae such as expansion of tumors and induction of diabetes, particularly those with underlying risk factors and known insulin resistance. However, the literature on clinical GH studies does not show any serious sequelae and those which have been reported appear more related to the gonadotrophin stimulation or ovarian responses, which may sometimes reflect hyperstimulation, even in patients categorized as poor prognosis where this is not related to a low ovarian reserve. A specific reaction to GH was reported in the Jacobs study of 1995 (7) with swelling of the hands and feet along with pain in the small joints of these areas. In our decade of experience with GH we have also noted this phenomenon, albeit in only a few women (∼2%) (41, 42). In each case the symptoms resolved over a few days once the GH injections were ceased. Despite the few adverse sequelae reported we would caution that case work-up requires the exclusion of tumors, particularly in the pelvis, abdominal cavity or breasts and fasting glucose undertaken to detect insipient diabetes. These aspects are part of our routine workup (49), particularly in light of the fact that ∼20% of our patients will progress to utilization of GH injections. So far, most protocols in assisted reproduction utilize low-dosage (1–4 IU daily) and only for short periods ranging 10–42 days. Where longer regimens are planned, women will require review of relevant clinical features and investigation review e.g., pelvic and abdominal ultrasound scanning, mammography and serum glucose studies as indicated. Reassuringly also, the pregnancy outcomes from GH-treated women appear perfectly normal from the perspective of both the obstetric features as well as the ensuing offspring. Our findings (41, 42) are strengthened by another substantial study (50). This international collaborative study from KIMS (Kabi/Pfizer International Metabolic database) reported by Vila and her colleagues in 2015 on 201 pregnancies where women ($n = 173$) or their husbands ($n = 28$) were treated with GH for hypopituitarism. The was no relationship between GH treatment and pregnancy outcomes. None-the-less, as the over-all direction of technical, physiological and clinical studies point to the idea that unexplained and poorly explained infertility is a reflection of, so far undiagnosed, AGHD, caution must be advised. In particular, the implications of observations on Laron dwarfism, which is an autosomal recessive disorder with mutations of GHR causing insensitivity to circulating GH. Serum levels of IGF-1 are consequently low, presumably from reduced hepatic production. Apart from dwarfism, such individuals have an increased sensitivity to insulin (reducing the risk of type-2 diabetes and reduced rates of all cancers. This implies that extending GH into older people may increase the problem of insulin insensitivity (causing more diabetes) and remove the low-GH protection effect from cancers and tumors (16). Some animal researchers have proposed that longevity and good health in advanced age is traded off against reproduction, the mechanism acting via somatotrophic signaling (51).

CONCLUSIONS

From a clinical perspective, this review article makes the case to consider that women requiring assisted reproduction and are classified as poor prognosis, may potentially be considered to have a subclinical degree of AGHD. In association with the rapidly increasing trend for delaying reproduction beyond age 35 years, GH is being widely researched now as a potential adjuvant for infertility treatment in this group who, studies consistently show, have a poorer prognosis than younger females when relying on autologous oocytes. The idea that the age-related reduction in fertility prognosis is a feature of GHD is supported by our, yet unpublished, studies showing an elevated binding protein IGFBP-3/IGF-1 ratio and this can be reduced to a normal range (matching younger, good prognosis women) by the administration of GH as an adjuvant.

In studies from different directions arising from its use as an adjuvant for IVF, it is likely that GH will be shown to have major enhancement effects on oocyte competence. Such studies should reveal major influences in the physiology of folliculogenesis and oocyte maturation. This will not only benefit older women but also younger women who currently have unexplained poor prognosis. We believe there is sufficient evidence to promote studies in two directions; firstly, to precisely define the subclinical AGHD condition among women attending fertility clinics; and secondly, to explore a more rationalized approach to the clinical use of GH. We would propose that studies should urgently be undertaken to assess whether IGF-I levels or IGFBP/IGF-I ratio can be a predictor of poor-prognosis. If so, then an RCT is required on naïve IVF cases to determine if GH adjuvant can provide a better chance for pregnancy and live birth in those predicted to have poor-prognosis.

Further studies are also required to determine appropriate and optimal dosage regimens. Currently most GH adjuvant use is applied concomitantly with the FSH-stimulation phase.

However, should the GH exposure begin much earlier, at the initiation of follicle recruitment and early oocyte activation? There is also a pressing need for studies to determine if GH can favorably influence the age-related effects on aneuploidy which is a reflection of diminished oocyte competency.

ETHICS STATEMENT

All protocols are endorsed under licence from the Reproductive Technology Council (RTC) of Western Australia (Practice Licence current to April 2021) as well as the Reproductive Technology Accreditation Committee (RTAC) under the auspices of the Fertility Society of Australia (accredited to April 2020). The reporting of any retrospective data analysis was provided by Curtin University Human Research Ethics Committee approval RD-25-10.

AUTHOR CONTRIBUTIONS

JY wrote the manuscript with contributions from research collaborators. SR for receptor studies. SZ for IGF-1 studies. KK for GH studies.

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REVIEW
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Growth Hormone and Insulin-Like Growth Factor Action in Reproductive Tissues

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The role of growth hormone (GH) in human fertility is widely debated with some studies demonstrating improvements in oocyte yield, enhanced embryo quality, and in some cases increased live births with concomitant decreases in miscarriage rates. However, the basic biological mechanisms leading to these clinical differences are not well-understood. GH and the closely-related insulin-like growth factor (IGF) promote body growth and development via action on key metabolic organs including the liver, skeletal muscle, and bone. In addition, their expression and that of their complementary receptors have also been detected in various reproductive tissues including the oocyte, granulosa, and testicular cells. Therefore, the GH/IGF axis may directly regulate female and male gamete development, their quality, and ultimately competence for implantation. The ability of GH and IGF to modulate key signal transduction pathways such as the MAP kinase/ERK, Jak/STAT, and the PI3K/Akt pathway along with the subsequent effects on cell division and steroidogenesis indicates that these growth factors are centrally located to alter cell fate during proliferation and survival. In this review, we will explore the function of GH and IGF in regulating normal ovarian and testicular physiology, while also investigating the effects on cell signal transduction pathways with subsequent changes in cell proliferation and steroidogenesis. The aim is to clarify the role of GH in human fertility from a molecular and biochemical point of view.

Keywords: estrogen, testosterone, granulosa cells, theca cells, Sertoli cells, Leydig cells, signaling

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INTRODUCTION

Growth hormone (GH) is a 191 amino acid protein, which binds readily to the growth hormone receptor (GHR) and in some species the prolactin receptor (1, 2). The GHR is a member of the cytokine receptor superfamily (3) and although the majority of human GHR has been detected in the liver, it has also been found to be abundantly expressed in all cellular components of the human ovary and testes (4, 5). GH was demonstrated to have both direct and indirect effects on ovarian and testicular function, with direct effects mediated by the explicit GH-GHR interactions, while indirect effects likely to be mediated through the local production of secondary factors, particularly Insulin-like growth factor (IGF) (6), a protein that is typically produced by the liver in response to GH stimulation (1, 7, 8). Both GH and IGFs form part of the somatropic axis, which is markedly active at onset of puberty, and responsible for whole body growth and development (9). At puberty, animals also become sexually mature, and it is clear that the somatropic axis is connected to the

establishment of reproductive function, but the precise mechanisms are still not fully understood (9, 10). While timing of puberty is genetically controlled (11), it is likely that the development of the body to a specific weight and/or size through the anabolic actions of GH and IGFs is at least partly responsible for onset of puberty (10, 12). This system is highly conserved from an evolutionary perspective, and has been observed across various animals including mammals and fish (10).

GH has been shown to have multiple specific effects in female and male reproductive physiology, such as promotion of steroidogenesis, enhancement of gonadotropin sensitivity as well as significant stimulatory effects on spermatogenesis and follicular development, which ultimately aligns with the initiation of puberty (4, 13, 14). This means that the GH-IGF system is likely to have profound effects on the major reproductive constituents of the ovary including granulosa cells, theca cells, and oocytes and in the testes including spermatids, Sertoli, and Leydig cells. Most of the biological understanding of the action of this system has been derived from animal studies, as access to developing human follicles from oophorectomy and testicular biopsy is limited. However, the specific biochemical interactions are under-researched. Nonetheless, we report here on the current knowledge regarding the biological and biochemical actions of both GH and the IGF system in female and male reproductive function, citing animal and where possibly, human studies. We will explore the effects of these proteins on follicular dynamics including growth and progression, proliferative effects on reproductive cells, production of key sex steroids such as testosterone, estrogen (E2), and progesterone (P4), the regulation by gonadotropins, and finally the intracellular signaling that mediate these activities.

GH, GHR, AND FOLLICULAR GROWTH

GH has been reported by many studies to modify the growth of developing ovarian follicles (15–17). *In vitro* studies using caprine preantral follicles have demonstrated the stimulatory effect of GH on antral follicle development particularly during the initial antral phase (15). GH exposure over 18 days increased the diameter of caprine preantral follicles, and using *in vitro* maturation protocols, led to the generation of healthy oocyte-cumulus complexes, production of more metaphase II oocytes, and better fertilization ability (15). The same investigators showed that GH exposure over a similar period functioned synergistically with Follicle Stimulating Hormone (FSH) in supporting canine follicular growth, increasing the follicular diameter, promoting viability, and it was suggested that this was due to GH-induced production of antral follicle fluid and consequential antrum formation (**Figure 1**) (16). This response was largely observed in a separate study in secondary bovine follicles exposed to GH for 32 days, where the follicle diameter, antrum formation and E2 release were all increased (17).

The expression status of GHR mRNA at different follicle developmental stages was investigated in the goat, and high expression was found in oocyte, stromal, cumulus and mural granulosa cells of both small and large antral follicles (18). Interestingly, GHR was not detected in preantral follicles, and this may imply that any effect in the earliest follicular stages is mediated indirectly, possibly through the local GH-induced production of IGF, but in later, more mature follicles, they may respond directly to GH stimulation via the expression of the GHR. This observation was supported by another study where an elevated number of primordial and atretic follicles were found in GHR knock-out mice. They also showed a decreased number of primary, secondary, antral, and healthy growing follicles indicating failed follicular progression possibly due to the inability to upregulate sufficient GHR as follicles develop (19). Importantly, follicle progression was corrected with IGF-1 treatment (19), but this IGF-mediated effect was not observed in all GHR knock-out murine studies (20). Other investigations using knock-out animal models have provided further evidence to indicate that GH influenced reproduction, but was not completely essential for generating offspring. For example, while the absence of functional GHR was reported to cause an increase in systemic GH levels, a decrease in circulating IGF-1 level (but still present), and a delay in puberty onset with a reduced number of ovarian follicles, these animals could still reproduce, but with a smaller litter size (21–24). Several studies have confirmed that GHR knock-out resulted in a delay in puberty onset, and this echoes the delayed puberty that is observed in human disorders such as Laron dwarfism where GHR is dysfunctional (25, 26).

Taken together, it is reasonable to assume that the GHR influences fertility given its effect on puberty and that GH supplementation can restore fertility in humans with GH-deficiency (27). Furthermore, since the GHR was expressed on all cellular components of female adult follicles, it stands to reason that they contain the necessary cellular machinery for mediating direct actions (4). Moreover, membrane bound GHR was also reported to be expressed on the human oocytes, which suggested that GH may act directly on the oocyte itself, as well as indirectly via granulosa cells (28). However, GHR expression was not evident in fetal oocytes, perhaps indicating that it becomes active later in development, although this could be an artifact related to fetal termination prior to ovarian tissue extraction (4). Nonetheless, either directly or possibly indirectly through IGF-1, GH was demonstrated to play major role in primordial follicular growth and progression in various animal models and consequently it may regulate the recruitment of primordial follicles into the growing, gonadotropin-sensitive pool (16, 18). This is possibly one reason as to why beneficial effects are observed with GH supplementation during IVF treatment (29, 30).

GH AND OVARIAN CELL PROLIFERATION, DIFFERENTIATION, AND GONADOTROPIN RESPONSE

There is evidence to suggest that GH and/or IGF act in synergy with gonadotropins, FSH and luteinising hormone (LH), in reproductive tissue to promote granulosa and theca cell expansion, along with granulosa cell differentiation to luteal

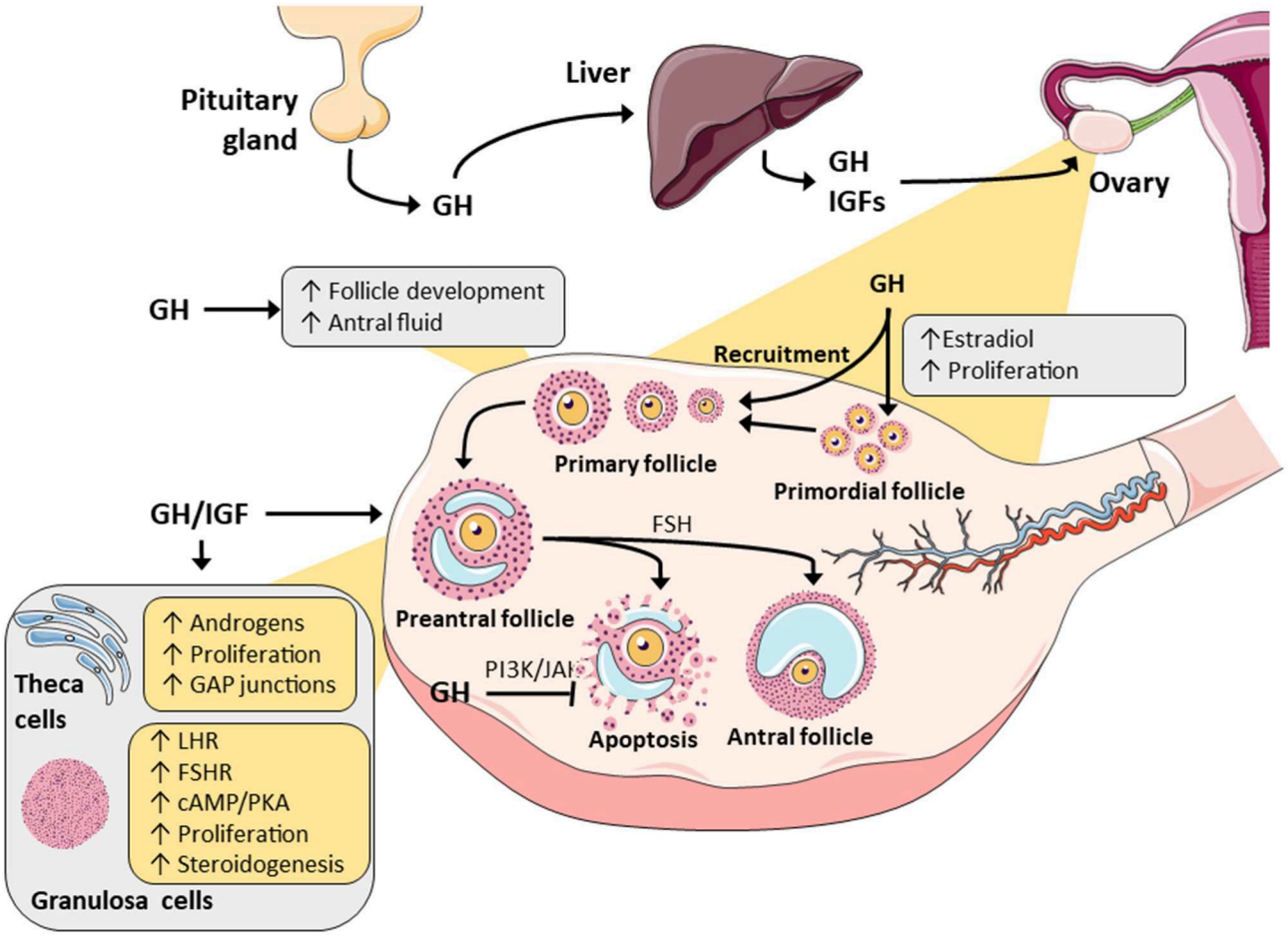

FIGURE 1 | A summary of the major actions of GH and IGF in ovarian physiology. Both have been demonstrated to promote steroidogenesis in granulosa and theca cells through alterations in metabolizing enzymes. GH/IGF have also been reported to synergistically work with gonadotropins to alter steroidogenesis and this is possibly mediated by changes in the gonadotropic receptors. Finally, through intracellular signaling pathways (JAK/STAT and PI3/AK), GH and IGF may promote follicle selection and survival by decreasing follicular atresia.

cells (**Figure 1**). In rat ovaries (31), GH treatment in the presence of FSH enhanced granulosa cell differentiation, but there was no change in cell proliferation. Conversely, in mouse follicles, addition of GH enhanced both granulosa and theca cell proliferation (**Figure 1**) (32). However, it has remained unclear whether GH induces cell expansion directly through GHRs expressed on these cells, or indirectly via stimulation of secondary growth factors produced by granulosa cells, such as IGF, which would then directly act on theca cells (32, 33). The GH-induced response in theca cell proliferation was confirmed in ovine *ex vivo* models, where the high concentrations of GH caused excessive growth of theca cells, such that they depleted nutritional elements in the medium (34). This effect was further corroborated in an *in vitro* study, where high doses of GH were found to be harmful to rat preantral follicle survival, possibly due to excessive theca and stromal cell proliferation and subsequent nutrient depletion (35). Due to these proliferative effects and expansion of follicular cells, the addition of GH to alginate-based growth media containing bovine secondary follicles produced higher levels of E2 synthesis and secretion (17). It was also noted that this increase in E2 production could contribute to the preservation of follicular architecture and function, and lead to better follicular development (17).

Interaction of FSH and LH with their complementary gonadotropin receptors (i.e., FSHR and LHR) induces downstream signaling that is critical for steroidogenesis, proliferation, and differentiation, and both signal through the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway to enhance production of E2 and P4 (**Figure 2**). FSHR and LHR are both G-protein coupled receptors (GPCRs) that transmit the intracellular cascade via adenylate cyclase activity, cAMP accumulation, with subsequent activation of PKA, which then phosphorylates the transcription factor CREB (cAMP response element binding protein). CREB binds cAMP response elements (CRE) in genomic DNA causing the transcription of various genes including those encoding for steroidogenic enzymes (e.g., aromatase) and cholesterol transport, the

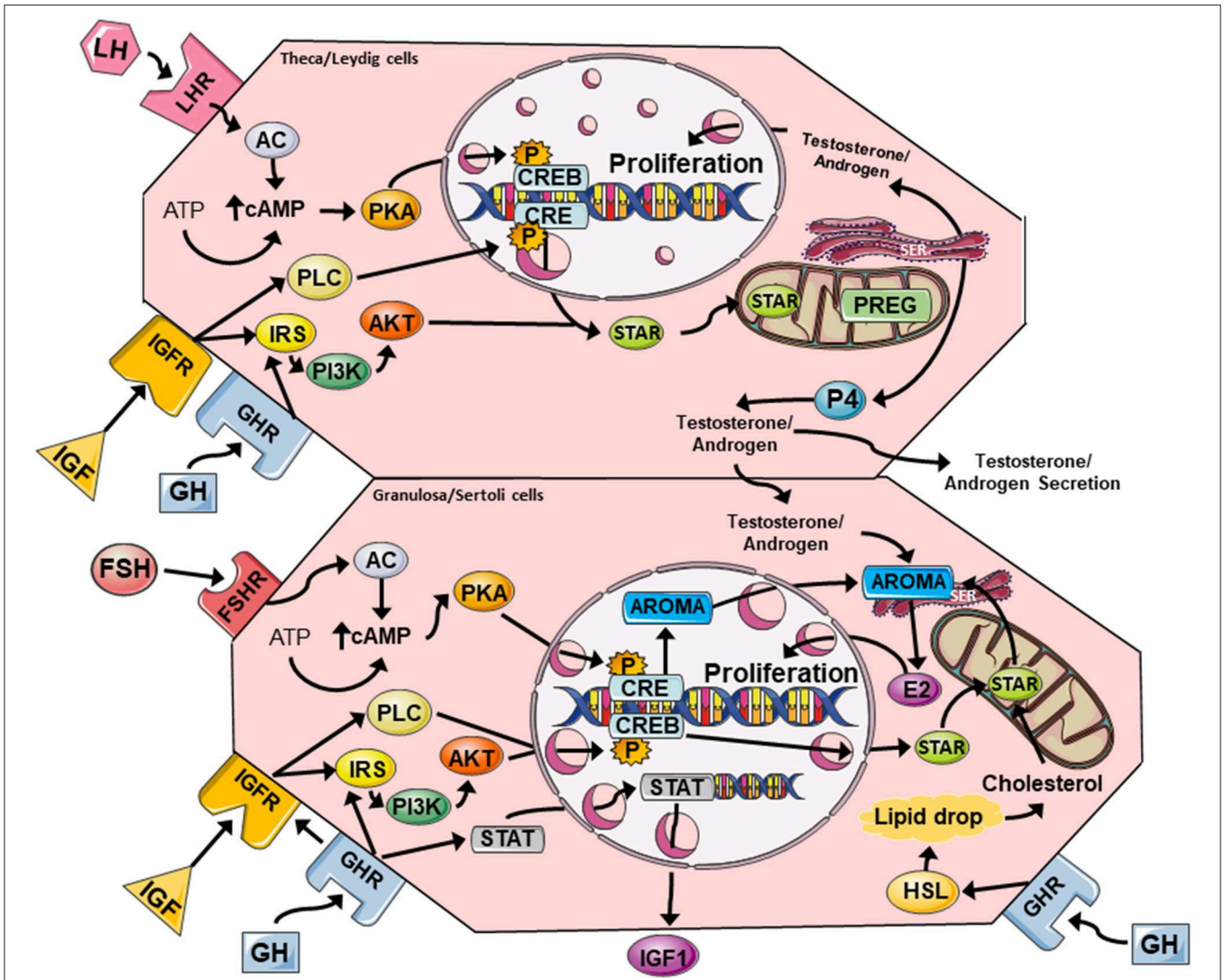

FIGURE 2 | A summary of the major GH and IGF signaling networks in female (theca/granulosa cell) and male (Leydig/Sertoli cell) reproductive physiology. Both GH and IGF can activate PLC/PKC and PI3K/Akt pathways that cross-talk with FSHR and LHR signaling via cAMP/PKA to promote steroidogenesis and cell proliferation. Steroidogenic events are mediated by CREB-dependent expression of aromatase (granulosa cells), and StAR expression in all cell types. StAR allows cholesterol to enter the mitochondria where it can be converted to PREG, and then subsequently to testosterone/androgens, estrogens, and progesterone. Estrogens and testosterone enhance cell proliferation via autocrine mechanisms, while GH can induce local IGF expression in granulosa and Sertoli cells via JAK/STAT signaling. LHR, luteinising hormone receptor; FSHR, follicle stimulating hormone receptor; AC, adenylate cyclase; cAMP cyclic AMP; PKA, protein kinase A; CRE, cAMP response element; CREB, cAMP response element binding protein; PLC, phospholipase C, IRS, insulin receptor substrate; PI3K/Akt, phosphoinositide 3-kinase/protein kinase B; StAR, steroidogenic acute regulatory protein; PREG, pregnenolone; SER, smooth endoplasmic reticulum; P4, progesterone; E2, estradiol; STAT, signal transducer and activator of transcription; AROMA, aromatase, HSL, hormone-sensitive lipase.

precursor substrate for sex steroid synthesis (e.g., steroidogenic acute regulatory protein, StAR).

The GH-GHR interaction in granulosa cells can modulate FSH action and also induce the expression of LHR (31, 36). This downstream expression of LHR is a key marker of granulosa cell differentiation to luteal cells, and can also possibly be influenced by GH stimulation of IGF within the ovary, which acts in a paracrine manner when it augments granulosa cell expansion (33, 37). The reported effect of GH on FSHR and LHR expression *in vitro* (31) and *in vivo* (38) is not trivial. This indicated that GH may modify or potentiate the sensitivity of granulosa cells and/or theca cells to gonadotropin stimulation and subsequently regulate sex steroid synthesis and release in follicles, which then boosts cell growth as paracrine/autocrine steroidogenic factors (**Figure 1**) (39). The two cell theory explains that ovarian steroidogenesis is regulated by consequent and mutually dependent processes (40), where LH stimulates theca cells to produce androgens, which are converted to various estrogens by the aromatase enzyme expressed in granulosa cells under the induction by FSH (40). Prior to

oocyte release, granulosa cells become luteinised by upregulating LHR expression, responding to human chorionic gonadotropin (hCG), and producing progesterone thus forming the corpus luteum in the secretory phase of the menstrual cycle.

GH was shown to promote androsterone and androgen synthesis in rat theca cells, and this response was independent of IGF production and cAMP accumulation (**Figures 1**, **2**) (6). In rat granulosa cells, co-treatment with FSH and GH significantly enhanced LHR expression and increased P4 synthesis and secretion, but there was no change in E2 production or cell proliferation (31). Central to these effects in granulosa cells was a clear enhancement of FSH-induced accumulation of cAMP, which is a key mediator of steroidogenesis, hormone receptor formation and differentiation of granulosa cells into luteal cells (41). Interestingly, *in vivo*/*ex vivo* studies in women with decreased ovarian reserve revealed that GH supplementation as part of IVF treatment, increased the expression of LHR, FSHR, and GHR in human granulosa cells isolated after egg collection (28, 38). GH also acted on supporting the maturation process of luteinization by increasing LHR density and by reducing the expression of FSHR prior to ovulation (38). The cytosolic accumulation of cAMP and activation of PKA signaling can also be triggered via GHR- and IGFR (insulin-like growth factor receptor) cross-talk, and this is likely to influence gonadotropin responses (42). The convergence of gonadotropin response and these pathway on steroidogenesis in female and male reproductive tissues is discussed below.

OVARIAN GH-IGF AXIS

Interaction of GH with GHR can activate canonical and non-canonical downstream signaling. In canonical signaling, pituitary GH stimulates liver cells to release IGF into the circulation through transcription factors activated by GH-GHR. The ligand-receptor interaction triggers recruitment and autophosphorylation of JAK2 (Janus kinase) at the cytoplasmic domain of the GHR, and the GHR/JAK2 complex subsequently phosphorylates STAT (signal transducer and activator of transcription) molecules (particularly STAT5a, 5b, 1, and 3), which translocate to the nucleus and modify gene transcription leading to significant effects on cell proliferation (43). STAT5b is of most importance and directly regulates the expression of IGF-1 (43, 44), and was demonstrated to be a mediator of GH-induced IGF-1 production in rat granulosa cells (**Figure 2**) (33, 45, 46). Non-canonical GH-GHR intracellular signaling is typically independent of JAK2, and involves recruitment of Src family non-receptor tyrosine kinases (45), stimulation of phospholipase Cγ (PLCγ) and via cytosolic calcium flux from organelles, activation of protein kinase C (PKC) (47). As explained later, these components can cross-talk with other pathways such as the MAPK/ERK1/2 (mitogen activated protein kinase/extracellular signal-regulated kinase 1/2) and PI3/Akt (phosphoinositide 3-kinase/protein kinase B) signaling cascades causing changes in gene expression and modifying cell metabolism and proliferation (47).

It is not obvious which hormone or system is more important, as ovarian function can be influenced by systemic GH and IGF, local GH, GH-induced local IGF, and/or GH-independent IGF (48). However, it is clear that the GH-IGF axis is a key growth factor system involved in folliculogenesis (49). GH was shown to increase IGF-1 mRNA expression in rat preantral follicles (50) and promoted IGF-1 secretion from ovine granulosa cells (51). Furthermore, addition of IGF binding protein-3 (IGFBP-3) antagonized the anti-apoptotic effects of GH, which suggested that exogenous GH promoted local IGF-1 production that prolonged follicular survival (51). Consequently, the interplay of the GH-IGF system in the ovary is complex, as it can be utilized in paracrine and autocrine processes by granulosa cells and does not necessarily require GH stimulation.

The systemic IGF system is made up of IGF-1 and IGF-2 (52), type 1 and type 2 IGF receptors (IGF-1R &−2R) and six IGF binding proteins (IGFBP 1-6) (53), that regulate IGF bioavailability (54). However, paracrine expression of these components is also important during ovine folliculogenesis, and the local level of IGF is enhanced by the decreased expression of IGF biding proteins (IGFBP-2,-3,-4,-5, and−6) in growing follicles, as they advance from potential atresia to follicle selection (55). Expression of IGF-2 was decreased in atretic follicles while IGF-2 R and IGFBP-5 was significantly increased in atretic follicles (55). These data indicated that reducing the local bioavailability of IGF leads to follicle demise simultaneously suggesting that IGF expression is key for follicle survival and possibly selection (54). The level of ovarian IGF is also related to the stage of folliculogeneiss, with low levels detected in theca cells derived from medium sized follicles and in oocyte from infants (56), while higher levels of IGF-2 expression were observed in granulosa cells isolated from large antral follicles (54). Therefore, it appears that there is a dynamic requirement for GH and IGF activity as follicles mature and grow.

Analogous to GH, IGF-1 stimulates proliferation and differentiation of granulosa cells and theca cells (57, 58). It does so by also potentiating FSH actions on granulosa cells, and it was demonstrated that the IGFR was absolutely required for FSH-mediated activation of the PI3K/Akt pathway which is a pro-survival cascade, and subsequent granulosa cell differentiation (59, 60). Zhao et al. found that the presence of IGF-1 stimulated cell proliferation in rat primordial follicles by measuring the increasing DNA content within the follicular cells (61). They also noted that cells cultured with IGF-1 exhibited better morphology due to the increased number of gap junctions between theca-granulosa cells and granulosa cell-oocyte (61). They detected 80% more cortical granules underneath the oocyte membrane with IGF-1 exposure, and hypothesized that it potentially accelerated the development of the preantral oocyte cytoplasm. In addition, the presence of FSH and IGF-1 improved preantral follicular growth due to the activation of the FSHR (61). The stimulatory effects of IGF-1 on follicular and cell survival have also been shown in caprine preantral follicles and oocytes (62, 63), in porcine granulosa cells (64) and in bovine antral follicles, oocytes and granulosa cells (65). An *in vitro* study performed by Magalhães-Padilha et al., demonstrated a higher growth rate of IGF-1 stimulated caprine preantral follicles and they

stipulated that it was most likely due to cellular proliferation, as it was demonstrated that IGF-1 enhanced nuclear maturation of granulosa cells in preantral follicles (63).

Animal studies using genetic knock-outs also demonstrated a more direct role for IGFs over GH in reproduction. For example, female mice with IGF-1R knock-out were shown to be completely sterile, with no antral follicles and a 90% reduction in serum E2 levels (66). In fact, inactivation of either IGF-1 or IGF-R by knock-out is incompatible with life in the majority of the cases, and in the rest of the cases, it certainly causes infertility in both sexes with an infantile reproductive system (67). Moreover, IGF-1 knock-out mice exhibited significantly reduced expression of FSH receptors and consequently reduced aromatase expression and E2 secretion (68), indicating that IGF-signaling may regulate gonadotropin receptor expression.

The combination of FSH and presence of the IGFR leads to various intracellular signaling events such as cAMP production, which as outlined in turn activates PKA and CREB, along with activation of the MAPK/ERK1/2 and PI3K/Akt pathways (60, 69). These signaling mechanisms increase aromatase activity and LHR expression (54). To induce aromatase activity, FSH, and IGF-1 or−2 work in synergy and act on their respective receptors (FSHR and IGF-1R) (60). IGF-1 has specific and stimulatory effects on granulosa cells, and it was reported to increase the expression of steroidogenic enzymes CYP11A1, 3β-hroxysteroid dehydrogenase (3βHSD), CYP19A1, along with IGF-1R, and FSHR gene expression (70). It was noted that IGF-1 activated steroidogenic and apoptotic regulatory genes through activation of PI3K/Akt pathway in bovine granulosa cells (70, 71). Both IGF-1 and IGF-2 can stimulate the production of sex steroids involved in follicular development. IGF-1 together with LH enhanced granulosa cell P4 production and acted as regulator of E2 synthesis in luteal cells (71). Importantly, the IGF-1R is also critical for the increased expression of StAR under FSH stimulation, which is required for mitochondrial transport of cholesterol for the first step of sex steroid synthesis, pregnenolone production (60). Furthermore, it has been demonstrated that high concentrations of GH/IGF supress the activity of Anti-Müllerian hormone (AMH), which is exclusively secreted in gonadal tissues (72). AMH is one of the members of transforming growth factor beta (TGF-β) super-family of growth factors, and downregulates both development and the function of preantral and antral follicles in primates (73, 74). This action may partially explain the role of GH/IGF in regulating follicular development and selection. Taken together, these data indicated clearly that IGF plays a central role in regulating follicular development via granulosa cell proliferation, differentiation, steroid production, and by mediating the stimulatory activity of gonadotropins. These effects along with that of GH are summarized in **Table 1**.

ROLE OF GH IN TESTES

Expression of GH has been detected across various systems in the human including neural, immune, respiratory, and reproductive tissues (84, 85). There are two clinically important versions of GH, including the normal GH form (GH-N) secreted from the pituitary gland, and the variant GH form (GH-V), first detected in the placenta (84). While both isoforms have been detected in the human testes, as well as in the testes of other mammals, GH-V was shown to be predominantly expressed form in human testes (86, 87). The level of testicular GH was found to be significantly less than that observed in the pituitary, and thus testicular GH is expected to act locally and not systemically (84). GH receptors and GH binding proteins were also observed to be abundantly expressed throughout the male reproductive system, in Leydig cells, Sertoli cells, seminal vesicles, epididymis, vas deferens, and prostate (5). However, the majority of the GH-induced effects on seminiferous tubules and testicular growth have been found to be indirect, and mainly accomplished via IGF generation and action (88, 89). Importantly, IGFR was reported to be expressed in the male reproductive tract including localization in early spermatids, secondary spermatids, Sertoli cells, and to a lesser extent Leydig cells (90, 91). Interestingly, men with distal chromosome 15 structural abnormalities are more likely to experience cryptorchidism, and this appears to involve the IGFR locus (92). Therefore, it is reasonable to expect that the GH-IGF axis could regulate aspects of male reproductive function and development.

GH is accepted to play an important role in sexual maturation in all mammalian species, and is an important contributor to the onset of puberty (14). Both in humans (93) and in experimental animals (94), pubertal delay was observed with GH deficiency (83). During male puberty, GH has roles in testicular development and differentiation, stimulation of germinal cell differentiation (79), influencing increased testicular diameter (8), and aiding in the development of the Wolffian ducts (89), all of which are underdeveloped in GH-knock-out mice (95). Furthermore, there is a positive correlation between serum IGF levels and testicular volume (95, 96), and administration of GH can significantly accelerate puberty if onset has been delayed (90, 97). In Laron dwarfism, due to the insensitivity to GH, and in GHR knock-out mice, appropriate testicular function develops later than in healthy males, but still occurred (88). This indicated that testicular development may be only partly GH-dependent, with the majority of stimulatory effects on testes mediated directly by IGF (88).

Moreover, gametogenesis is significantly influenced by GH. Ovesen et al. demonstrated an increase in sperm motility in GH-treated men and an increase in semen volume in oligospermic men treated with GH (78). In addition, it was found that GH supplementation caused an increase in germ cell number and an improvement in sperm morphology (82, 88). The potential mechanism by which GH may improve spermatogenesis is possibly through the stimulation of Leydig and Sertoli cell differentiation (14). Furthermore, GH was found to improve Leydig cells responsiveness to physiologic human chorionic gonadotropin (hCG), a key hormone regulating spermatogenesis (8).

When GH-deficient rats were treated with GH, it was demonstrated to have a protective effect on the count and motility of spermatids following treatment with cyclophosphamide (98) In addition, it prevented testicular atrophy and testosterone depletion after treatment with methotrexate (88, 99). Both agents

TABLE 1 | Summary of major findings from GH and IGF studies in ovarian and testicular physiology.

Gender/Factor	References	Model/Tissue type/Cell type	Major effects mediated by factor	
FEMALE				
Growth hormone				
	Araújo et al. (17)	Bovine follicles	↑ Antrum formation	↑ Estadiol concentration	–
	Sirotkin and Makarevich (33)	Bovine granulosa cells	↑ IGF-I secretion	↑ IGFBP-3 secretion	↓ Presence of regulatory PKA subunit
	Serafim et al. (16)	Canine follicles	↑ Antrum formation	↑ Estradiol secretion	↑ Follicular diameter
	Magalhaes et al. (15)	Caprine follicles	↑ Antrum formation	↑ M2 oocyte yield	↑ Nuclear maturation
	Martins et al. (18)	Caprine ovaries	↑ Development of preantral follicle	GHR mRNA detected in antral follicles	GHR mRNA not present in preantral follicles
	Weall et al. (28)	Human COC oocytes	↑ Oocyte mitochondrial function	↑ oocyte quality	GHR detected on human oocyte
	Regan et al. (38)	Human granulosa cells	↑ Density of FSHR, BMPR1B, LHR, and GHR	–	–
	Kobayashi et al. (32)	Murine preantral follicles	↑ Granulosa cell proliferation	↑ Theca cell proliferation	–
	Arunakumari et al. (34)	Ovine preantral follicles	↑ Development of preantral follicle	↑ Nuclear maturation of the oocyte	–
	Khalid et al. (51)	Ovine granulosa cells	↑ IGF-I secretion	↑ Estradiol secretion	↑ porgesterone secretion
	Apa et al. (6)	Rat theca cells	↑ Androstendione sythesis	↑ Androgen production	–
	Jia et al. (31)	Rat granulosa cells	↑ FSH-stimulated LH receptor count	↑ FSH-stimulated progesteron secretion	↑ FSH-stimulated 20 alpha-hydroxy-4-pregnen-3-one secretion
	Eisenhauer et al. (50)	Rat preovulatory follicles	↓ Follicle cell apoptosis	↑ GH-induced IGF mRNA	–
	Zhao et al. (35)	Rat preantral follicles	↑ Growth of preantral follicle	↑ Morphology quality of preantral follicle	–
			↑ Presence of catalytic PKA subunit	↓ Progesterone secretion	↓ Apoptosis incidence
Insulin-like growth factor				
	Walters et al. (65)	Bovine antral follicles	↑ Follicular size	↑ Estradiol secretion	↑ oocyte health
	Mani et al. (70)	Bovine granulosa cells	↑ Proliferation	↑ Estradiol secretion	↑ CYP11A1, HSD3B1, CYP19A1, BAX, IGF1R and FSHR expression
	Zhou and Zhang (62)	Caprine preantral follicles	↑ Proliferation	↑ Preantral follicle survival rate	-
	Magalhaes-Padilha et al. (63)	Caprine preantral follicles	↑ Percentage of normal follicles	↑ Rate of antrum formation	↑ Meiotic resumption rates
	Baumgarten et al. (60)	Human cumulus granulosa cells	↑ Proliferation	↑ Differentiation	PI3K/AKT mediated
	Zhou et al. (68)	Murine ovary	↑ Granulosa cell FSHR expression	–	–
	Hastie and Haresign (55)	Ovine ovary	↑ IGF-2 in large follicles	↓ IGF-II in atretic follicles	↑ IGFBP-5 in artretic follicles
	Campbell et al. (58)	Ovine & bovine granulosa cells	↑ Cell proliferation	↑ Oestradiol secretion	–
	Guthrie et al. (64)	Porcine granulosa cells culture	↓ Spontaneous apoptosis	–	–
	Zhao et al. (61)	Rat preantral follicles	↑ Follicular diameter	↑ Folicular morphology	↑ Cortical granules
MALE				
Growth hormone				
	Sjogren et al. (75)	*In vivo* canine treatment	↓ Testicular and prostatic weight	–	–

(Continued)

TABLE 1 | Continued

Gender/Factor	References	Model/Tissue type/cell type	Major effects		
	Matsushima et al. (76)	*In vivo* GH/thyroxine deficient mice	↑ Seminiferous tubule cell count	↑ Sperm count	↓ FSH levels
	Piotrowska et al. (77)	Murine testes	↑ Testicular size	↑ Testicular aging	↓ LHR & AR
	Ovesen et al. (78)	*In vivo* humans	↑ Serum/seminal IGF-I and serum IGFBP-3	↑ Sperm motility	↑ IGF
	Arsenijevic et al. (79)	*In vivo* rat testicles	↓ Testicular and seminal vesicle size	↓ Spermatogenesis	-
	Kanzaki and Morris (80)	Rat Leydig cells	↑ Androgen production	↑ StAR activity	↑ 3β-HSD mRNA expression
Insulin-like growth factor					
	Dance et al. (81)	Bovine Sertoli cell culture	↑ Cell proliferation	–	–
	Bingol-Kologlu et al. (82)	Murine germ cells	↑ Haploid cell number	–	–
	Saez et al. (83)	Porcine Leydig and Sertoli cell lines	↑ Stimulatory effect of FSG on cAMP production in Sertoli cells	↑ Pregnenalone to testosterone conversion	↑ Plasminogen activator

are important chemotherapeutics used in cancer treatment, and thus GH supplementation may play a role in preservation of fertility with cancer therapy.

Male GH knock-out mice have a significantly lower cell number in seminiferous tubules, which with corresponding underdevelopment of sperm, decreases fertility (76). The effects of GH on testicular development are evident in its stimulatory action on Leydig cell maturation and proliferation (77, 80). GH promoted androgen production along with StAR and 3β-HSD expression in progenitor Leydig cells (80). It was suggested that this action was mediated by activation of STAT5-dependent steroidogenesis by GH and through stimulation of Leydig cell proliferation (14, 80), although other studies in animals failed to demonstrate any androgenic effect (88, 100). In the latter situation, it could be that GH-mediated enhancement of aromatase activity leads to an increase in testosterone to E2 conversion, reducing testosterone levels but increasing E2 (72, 88). However, administration of GH and subsequent GH-mediated effects were found to be dose dependent, as high concentrations given to GH-deprived canines caused atrophy of testes and accessory organs, thinning of prostatic epithelium and a reduction in LH and testosterone levels (75). In addition, overexpression of GH in mice led to early testicular aging characterized by lower expression of the androgen receptor (AR) and LHR (77). Conversely, GH deficit does not supress ongoing spermatogenesis, although GH treatment has the ability to restore inhibited spermatogenesis possibly indicating a potentiating function (101). These findings indicate that the response of male reproductive organs to GH is complex.

ROLE OF IGF IN TESTES

Interestingly, GH-induced IGF secretion by Sertoli cells was reported to increase the number of LH receptors in Leydig cells, meaning that IGF could increase testicular androgen production (8, 83, 90), and both IGF-1 and IGF-2 were shown to enhance testosterone production (90). IGF also has proliferative actions on Sertoli cells in the same autocrine manner (90). As stated before, GH-induced effects on seminiferous tubules are mainly accomplished through IGF action (88, 89). IGFR expression has been shown in porcine Leydig and Sertoli cells (83). The majority of the IGF-1 effects on Leydig cells was found by examining knock-out mice. IGF-1 knock-out mice exhibited significant stunting in the development of vas deferens, seminal vesicles and prostate, along with developmental delay of Leydig cells, which were fewer than normal (67, 102). In addition, testosterone levels were reduced by 82%, and LH-stimulated testosterone production was decreased (67). However, capacitated sperm from these mice were able to fertilize oocytes (67). Interestingly, IGF regulates Leydig cell differentiation and stimulates hCG-dependent cAMP synthesis in order to stimulate steroidogenesis (90, 103). hCG was also shown to upregulate the expression of IGFR in rat Leydig cells suggesting cross-talk between both pathways (90, 103). Furthermore, IGF was demonstrated to increase responsiveness of porcine Leydig cells to physiological

hCG concentrations and to pharmacological steroidogenesis activators (83).

In cattle, IGF induced proliferation of Sertoli cells by 18% and was crucial for maintaining the Sertoli population (81). This stimulatory effect was enhanced significantly when IGF functioned in unison with FSH, echoing the response observed in female granulosa cells (81). It has been reported that IGF promoted thymidine inclusion in DNA of Sertoli cells and to have role as a mitogenic stimulator in immature Sertoli cells (8). Furthermore, it can regulate glucose and lactate metabolism in Sertoli cells, which are crucial metabolites for germ cell health (95). It also stimulates plasminogen activator production in Sertoli cells (8, 83, 95), which is secreted by Sertoli cells and plays an important role in germ cell development, formation, and migration (104). These effects in testicular biology, along with that of GH are summarized in **Table 1**.

GH-IGF SIGNALING: CONVERGENCE WITH GONADOTROPIN SIGNALING

While male and female reproductive systems are clearly different, the response to gonadotropins including cell proliferation and sex steroid synthesis/release is largely biochemically similar (**Figure 2**). It is likely that any effects GH and/or IGF have on male and female reproductive physiology is mediated through changes in these pathways, and potentiation of subsequent steroidogenesis, the products of which have their own effects on cell proliferation and survival. In this final section, we describe the intersecting biochemical points of gonadotropin and GH/IGF signaling.

Stimulation of the FSHR by FSH leads to activation of the cAMP/PKA pathway and subsequent CREB-mediated transcription of various genes. This process can upregulate the expression of steroidogenic genes such as those encoding aromatase and StAR, along with the LHR gene (105, 106). The aromatase gene is directly regulated by CREB (107), and this enzyme converts androgens (e.g., testosterone) to estrogens, while StAR mediates the transport of cholesterol substrate to the mitochondria for synthesis of testosterone, E2 and P4 in steroidogenic cells (**Figure 2**). However, the activation of FSHR and LHR GPCRs will trigger other key cell signaling events, that can also impact on steroidogenesis. One central pathway is the PI3K/Akt cascade, which is a well-known regulator of cell metabolism, proliferation and survival (108), and can be directly stimulated by FSHR following direct interaction with 14-3-3τ adaptor proteins (109). The PI3K/Akt pathway is also stimulated by LH, with its activity heightened in the presence of FSH (110, 111).

Akt is a multifunctional signaling hub that can regulate cell metabolism, proliferation, and death (36, 95, 112). FSH-mediated activation of Akt is essential for the expression of 3β-HSD, α-inhibin, CYP19, LHR (113), and there is accumulating evidence to indicate that FSHR-mediated aromatase expression requires both cAMP/PKA and PI3K/Akt activation (59, 60, 114). Interestingly, recent research in human and rodent granulosa cells has shown that intact IGF-1R signaling was also required for FSHR-mediated phosphorylation of Akt (66, 113). It is beginning to emerge that FSHR action requires obligatory PI3K/Akt signaling, and achieves this by supporting IGF-IGFR stimulation of Akt. This is evident from studies showing that FSH could not promote CYP19, LHR, and StAR expression in the presence of an IGF inhibitor (113). GH and IGF intracellular signaling are both connected to FSHR and LHR signaling via the regulation of the insulin signaling pathway which incorporates the PI3K/Akt cascade. IGF and proinsulin share homology such that both of their respective receptors, IGFR and the insulin receptor (IR), will bind to the alternate growth factors albeit with reduced affinity (3). Interaction of IGF with the IR leads to the recruitment and phosphorylation of insulin receptor substrate 1 or 2 (IRS1 or 2) with subsequent activation of PI3K then Akt (3). IRS1/2 are possibly the key intermediates between FSH and IGF-PI3K/Akt activation, as it has recently been proposed that in Sertoli cells, PKA stimulation by FSH leads to enhanced activity of protein phosphatase 1 (PP1), which can promote IRS signaling by dephosphorylating inhibitory serine/threonine residues (95). However, further studies are required, particularly in male reproductive organs, to confirm if this mechanism exists.

The vast majority of GH actions are mediated through the JAK-STAT signaling events which has multiple complex roles, such as regulating cell proliferation and oocyte maturation (**Figure 1**) (115, 116) and significant downstream crosstalk with the other pathways (42, 117). GH is likely to be involved in the above process simply by its ability to upregulate local IGF production through classical JAK/STAT signaling, but JAK2 can also directly cross phosphorylate IRS1/2 adding another link to the GH-GHR cascade (33, 118, 119). GH-GHR initiation of STAT5b can promote the expression of local IGF which then acts in an autocrine manner to stimulate PI3K/Akt signaling and enhances FSH-FSHR activities (**Figure 2**). Importantly, outside of JAK-STAT and PIK3/Akt, the GH-GHR, IGF-IGFR, IGF-IR interactions can stimulate several other different intracellular signaling cascades notably PLC/PKC and MAPK/ERK1/2 pathways (120).

A key aspect of GH and IGF stimulation of the PLC/PKC pathway, is the ability to promote CREB-mediated transcription. For GH-GHR, non-canonical intracellular signaling, which is independent of JAK2 involves recruitment and activation of Src family non-receptor tyrosine kinases (45). Src family molecules such as Shc and Lyn, interact with the cytoplasmic domain of the GHR and activate phospholipase Cγ (PLCγ) which then proceeds to hydrolyse phospholipids to form inositol-1,4,5 triphosphate (IP3) and diacylglycerol (DAG) (47). These components go on to increase cytosolic calcium flux from organelles and activate PKC, respectively. PKC activity is critical because it can also trigger CREB-mediated gene transcription directly, and thus StAR and/or aromatase expression for example (121). It was shown that stimulation of PKC led to enhanced StAR expression and progesterone secretion in Leydig and granulosa cells (**Figure 2**) (121, 122). For IGF, the IGFR can directly activate PLCγ also leading to the above signaling cascade. Interestingly, the PKC pathway can also be activated by FSHR via formation of IP3 and DAG and this leads to the expansion of cumulus cells and meiotic maturation of oocytes (123), again neatly demonstrating

crosstalk between gonadotropin GPCRs and GH/IGF signaling., The interconnection of these signaling systems at least partly explains the physiological effects observed *in vitro*/*ex vivo*.

One final convergence point of these related signaling events, is the initiation of the p38 MAPK and MAPK/ERK1/2 signaling pathways, that causes changes in gene expression and can modify cell metabolism and proliferation (47). ERK1/2 functions to enhance mitogenic signals in cells and can be activated by elevated intracellular calcium (from PLC/PKC events), and indirectly via PKA, both of which as outlined are stimulated by GH and IGF activity (106, 124). In granulosa cells, p38 MAPK plays a role in generating pro-apoptotic signals (124). In Sertoli cells, PKA stimulates MAPK/ERK1/2 and this leads to FSH-induced cell proliferation (125). However, the contribution of the MAPK/ERK1/2 signaling pathway to steroidogenesis is less clear. For example, MAPK activation is important for FSH-mediated StAR and progesterone synthesis, while blocking this cascade increases aromatase activity and E2 production (106, 126). However, in another study, IGF-mediated stimulation of progesterone synthesis and secretion in human ovarian cells is dependent on MAPK/ERK1/2 and p38 MAPK signaling (127). Both MAPK/ERK1/2 and the pro-survival PI3K/Akt pathways are stimulated by IGF-I, IGF-2, and activated IGF-1R in cumulus granulosa cells (60). Thus, this interplay and individual participation of MAPK signaling in steroidogenesis remains unclear.

FUTURE GH-IGF MECHANISMS TO EXPLORE AND CONCLUDING REMARKS

Since steroid hormones are not stored in large quantities in steroidogenic tissues, there is constant demand for cholesterol, the main precursor of steroidogenesis (128). Consequently, steroidogenic cells have numerous, small lipid droplets that contain cholesteryl esters that release free cholesterol upon stimulation by hormones. Two enzymes are important for liberating cholesterol, hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). HSL was found to be expressed in internal and external theca cells as well as granulosa cells of preantral follicles (129). ATGL was detected in granulosa and Leydig cells (128, 130). Both enzymes are responsible for 90% of lipolysis, and the activity of HSL is mediated through phosphorylation at various serine residues. PKA which is active in response to FSH, stimulates HSL activity via phosphorylation at Ser-563, Ser-650, and Ser-660 (42, 130). It was also observed that GH increases HSL mRNA and protein expression in mice, illustrating the direct regulatory role of GH in lipolysis (42, 131, 132). Conversely, it has been reported that GH may indirectly enhance ATGL expression *in vivo* through an unknown mechanism, but the effects of ATGL in steroidogenic cells in the ovary and testes requires further research (133). In catabolic conditions, the PLC, PKC, and MAPK/ERK signaling cascades play an important role in activating HSL and releasing lipids for energy production and as outlined, both GH and IGF can activate these pathways (46). Interestingly, it has been shown that ERK can directly phosphorylate HSL at Ser-600 increasing the enzyme activity in adipocyte cell lines (42, 134). GH and IGF have the ability to alter the direction of lipid metabolism via regulation of these enzymes and this may have important implications for lipid homeostasis in steroidogenic cells and tissues, especially those derived from the reproductive system where GH is used regularly as an adjuvant for fertility treatment. However, little research has explicitly explored this area in reproduction.

The downstream signaling from FSH-FSHR interactions is clearly central to the life and death balance observed in granulosa cells and developing follicles, and seems to have parallels in male reproductive cells. Some reports have indicated that altered FSH-signaling and/or over expression of FSHR can actually promote apoptosis in unselected follicles, which may potentially happen via excessive accumulation of cAMP or activation of p38 MAPK pathways (106, 135). It could be the case that the pro-survival signals mediated by GH and/or IGF, prevent pro-apoptotic events, and thus have a largely beneficial effect on male and female reproductive cell proliferation. The downstream FSHR and LHR signaling cascades are very diverse, but it is clear that there is significant cross-talk with pathways associated with GH and IGF signaling. GH is regularly used as an adjuvant in fertility treatment, and studies in animal and *ex vivo* human models demonstrate that GH and IGF regulate steroidogenesis, cell proliferation, and follicular development. While this area of research has undoubtedly progressed, it is still not completely clear which biochemical mechanisms are involved.

AUTHOR CONTRIBUTIONS

The concept of this review was designed by JY and KK. The initial manuscript draft was undertaken by EI and KK. VC, JK, and JY contributed substantially to manuscript revision. **Figure 1** was designed by EI, KK, and VC. All artwork produced by VC.

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The Effect of Growth Hormone on the Clinical Outcomes of Poor Ovarian Reserve Patients Undergoing *in vitro* Fertilization/Intracytoplasmic Sperm Injection Treatment: A Retrospective Study Based on POSEIDON Criteria

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The aim of this retrospective analysis is to explore whether growth hormone (GH) pretreatment is beneficial for patients with poor ovarian reserve undertaking *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) treatment. Poor ovarian reserve patients with anti-Mullerian hormone (AMH) <1.2 ng/mL were recruited and divided into the GH adjuvant group (GH+ group) and the counterpart without GH pretreatment (GH- group). One-to-one case-control matching was performed to adjust essential confounding factors between the GH+ group and GH- group. A total of 676 cycles were included in the present study with 338 cycles in each group. Conventional ovarian stimulation protocols were applied for ART treatment. Patients were further divided into POSEIDON group 3 (PG3, age <35 years) and POSEIDON group 4 (PG4, age ≥35 years), based on POSEIDON criteria. The demographic data, cycle characteristics, and clinical outcomes between the GH+ group and GH- group, as well as in the further stratified analysis of PG3 and PG4 were compared. GH adjuvant showed a beneficial effect on the ovarian response and live birth rate in poor ovarian reserve patients. Further stratification revealed that in PG4, there was a significantly increased number of good-quality embryos in the GH+ group compared to the GH- group (1.58 ± 1.71 vs. 1.25 ± 1.55, $P = 0.032$), accompanied by a reduced miscarriage rate and a greatly improved live birth rate (29.89 vs. 17.65%, $P = 0.028$). GH adjuvant failed to promote the live birth rate in PG3. In conclusion, GH pretreatment is advantageous by elevating ovarian response and correlated with an improved live birth rate and reduced miscarriage rate in POSEIDON poor ovarian reserve patients older than 35.

Keywords: growth hormone, poor ovarian reserve, poor ovarian responders, clinical outcome, POSEIDON criteria

INTRODUCTION

The incidence of low ovarian reserve among patients requiring assisted reproductive technology (ART) has been dramatically increasing. However, low ovarian reserve refers to depletion of the quantity and quality of oocytes in the ovary (1), and these patients may experience poor ovarian response, which is still a conundrum for clinicians (2). Many *in vitro* fertilization (IVF) centers supplement patients with various adjuvant therapies to enhance IVF success rates, such as growth hormone (3), coenzyme 10 (4), arginine (5), and dehydroepiandrosterone (6). The true beneficial effects of these therapies are actively debated (7). GH as an adjuvant therapy in IVF treatment has received most attention, such interest being resurrected by several interesting reports particularly since the mid-2000s (8, 9).

GH works through the somatotropic axis, which comprises GH, IGF-1, IGF-2 and their binding proteins and receptors. It has been proven to affect follicular recruitment directly or indirectly through insulin-like growth factor-1 (IGF-1) (10). The GH receptor has been shown to be expressed in theca cells, granular cells, and oocytes, both in animals and humans, providing physiological evidence for its efficacy in enhancing ovarian response and improving oocyte quality (11–13). Research on animals confirmed the indispensable role of GH in the various stages of follicular development, including follicle recruitment, development of preantral follicles, and gonadotropin sensitivity of antral follicles (11, 14, 15). GH binds to their receptors on granular cells resulting in enhanced proliferation and differentiation of the target cell (10). GH addition might elevate the density of FSH receptors in granulosa cells and increase the mitochondrial amount in human oocytes, which may further improve the ovarian response as well as the capacity to repair DNA mistakes (13, 16).

With these laboratory foundations, clinicians have increased confidence in GH adjuvant therapy for patients with POR, and many clinical studies have been performed. A prospective observational study of GH co-treatment with antagonist protocol reported advanced pregnancy and an increase in the number of high-quality embryos in the GH+ group (17). A recent multicentric randomized placebo-controlled trial, published by Norman et al. (18), was unable to demonstrate an increase in the live birth rate from the co-administration of GH in poor ovarian responders (PORs), but the study failed to reach its planned recruitment numbers (being only 130 instead of 390 cases). However, a large amount of literature did not reach a consensus on the effect of GH on PORs, with some papers reporting encouraging results (8, 9, 13, 19) and other articles posing negative consequences (20–22). There is no consensus on the dosage of GH treatment, the reported dosage is ranged from 1 IU every other day to 10 IU daily. 2 IU daily is the most economical and effective dose based on the combination of treatment experience in children with GH deficiency and IGF-1 levels among people of different ages, which is also confirmed in our previous study (9, 23, 24). In addition, the definition of POR is inconsistent and has more than 41 different visions in a recent meta-analysis enrolling 46 RCTs (25), which makes it difficult to determine the effect due to the heterogeneity of cases. Despite the recognized heterogeneity, there is a tendency to believe that GH addition may be beneficial for oocyte quality and thereby improving the live birth rate in the elderly subgroup.

Numerous definitions of POR in the past impede the consistency of research subjects in separate reports. In 2011, the European Society of Human Reproduction and Embryology working groups proposed the "Bologna Criteria" for POR (26). The effect of GH adjuvant in POR diagnosed by the "Bologna Criteria" still differs within the new research in that there are different durations and dosages of GH and mixed groups of patients. The shortcoming of this definition may be the very heterogeneity in patients with a disparate probability of successful conception (2), which was amended by the recently proposed POSEIDON (Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number) criteria for patients undergoing IVF (27). In short, patients were divided into two categories according to the POSEIDON criteria: patients with normal ovarian reserve (anti-Müllerian hormone [AMH] $\geq$ 1.2 ng/ml, antral follicular count [AFC] $\geq$5) and with unexpected poor or suboptimal ovarian response, patients with poor ovarian reserve (AMH <1.2 ng/ml, AFC <5) and with expected poor ovarian response. Both categories were further classified by age (POSEIDON group 1 [PG1] and PG3 <35 years, PG2 and PG4 $\geq$35 years) (27). The POSEIDON criteria stratifies low prognosis groups into more homogenous sub-groups and provides recommendations for clinical handling, which might be a better sorting scheme.

In the current retrospective report, we aimed to explore the efficiency of GH for patients in the specific cohort with poor ovarian reserve (AMH <1.2 ng/ml), including POSEIDON group 3 (PG3) and 4 (PG4). We examined whether GH could improve ART success rates by reducing miscarriage rates and thereby improving the live birth rate. Importantly, the study facilitates the exploration of the potential mechanism by which GH adjuvant may exert its benefits.

The study was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-Sen University (2016ZSLYEC-061).

MATERIALS AND METHODS

Patients

Poor ovarian reserve patients (AMH <1.2 ng/ml), who met the PG3 or PG4 criteria, and underwent ART treatment in the Reproductive Medicine Center of The Sixth Affiliated Hospital of Sun Yat-Sen University from January 2014 to April 2016 were enrolled. In the GH+ group, 2 IU of GH in the form of Jintropin (Gensci, Changchun, China) was administered during the preceding menstrual cycle on days 2–3, which included daily injection over a 6-week period in the lead-up to ovum pick-up (OPU). Other enrolled patients without adjuvant treatment were included in the GH- group. The exclusion criteria were: abnormal chromosome, hydrosalpinx, endometriosis, hyperprolactinemia, thyroid diseases, uterine disorders that affected embryo implantation, severe oligoasthenozoospermia or azoospermia of the male partner. Among the cycles included, one-to-one case-control matching was performed to adjust essential confounding factors between the GH+ group and GH-

group with SPSS 22.0, including age, AMH, body mass index (BMI) and AFC. A total of 338 cycles in the GH+ group and 338 cycles in the GH- group were enrolled in the data analysis. The demographic data, cycle characteristics and clinical outcomes of the GH+ group, were compared with their counterparts in the GH- group.

Protocol for Controlled Ovarian Hyperstimulation (COH)

2 IU of GH daily in the form of Jintropin (Gensci, Changchun, China) was given subcutaneously on days 2–3 of the preceeding menstrual cycle until ovum pick-up (OPU) in the GH+ group. Conventional protocols, including both the antagonist and the long agonist protocol were applied in both groups (noting that the 2019 ESHER COS guideline indicated that these were equally effective for poor responders) (28). In the gonadotropin-releasing hormone (GnRH) antagonist protocol, gonadotropin (Gn) was administered from the second day of the cycle, and GnRH antagonist was administered subcutaneously daily when the leading follicle reached 14 mm until the day of hCG trigger. In the GnRH agonist protocol, 0.1 mg/day of leuprolide acetate was given subcutaneously from the midluteal phase of the previous cycle and Gn was administered 14 days later at the same time after achieving desensitization (FSH <5 IU/L, LH <5 IU/L, E2 <50 pg/ml) until the day of hCG administration. Ovidrel (Merck Serono, Germany) 0.25 mg was injected for the final trigger when dominant follicles reached 16 mm in diameter. Ultrasound-guided oocyte retrieval was performed ∼36 h after the trigger.

Embryo culture was performed following standard protocols and scored by the international morphological grading system, Peter scoring system: grade 1, blastomeres are almost even with no particle cytoplasm, fragmentation rate is <5%; grade 2, blastomeres are slightly uneven with cytoplasm contained some particles, fragmentation rate is between 5 and 20%; grade 3, blastomeres are obviously uneven with obvious particles in cytoplasm, fragmentation rate is between 21 and 50% and grade 4 refers to embryos that blastomeres are severely uneven with severe particles in cytoplasm and the fragmentation rate is more than 50% (29). Cleavage embryo on day 3 with grades 1–3 and at least 5 blastomeres are considered as transferrable embryos, and cleavage embryo grades 1 or 2 with 6–10 blastomeres were considered as good quality embryos. Blastocysts were evaluated with the Gardner scoring system: grading stage 1–6 by the expansion and hatching of the blastocyst; rating A-C for the inter cell mass (ICM) and trophectoderm (TE) (30). Blastocysts on day 5/6 with stage 2–6 are considered to be embryos suitable for transfer, and scoring 3BB or higher are considered as good quality embryos. Fresh embryos were transferred either on day 3 at cleavage stage or on day 5 at blastocyst stage, no more than 2 embryos, were transferred. The luteal phase was supported by Utrogestan (Besins, France) 200 mg vaginally twice a day starting on the day of OPU.

Data Collection and Outcome Measures

Data including age, duration of infertility, AFC, basal FSH, AMH, total Gn dosage, Gn duration, endometrium thickness, embryo development, and clinical outcomes were compared. Serum β-HCG levels >50 U/L at 12 days after blastocyst transplantation or at 14 days after cleavage stage embryo transplantation were confirmed as chemical pregnancies. Clinical pregnancy was identified by a gestational sac 3 weeks after a positive hCG test. The miscarriage rate was computed as the number of cycles that resulted in miscarriage by the number of clinical pregnancy cycles. The implantation rate was calculated as the number of gestational sacs divided by the number of embryos transferred. The live birth was defined as each live delivery of at least one fetus after 28 weeks of gestation.

Statistical Analysis

A 1:1 case-control matching undertaken as a computer-generated exercise, was carried out to match the essential confounding parameters [age, AMH, body mass index (BMI), AFC] between the GH+ and GH-groups. The results are expressed as the mean ± standard deviation (SD) for numeric variables and the percentages for categorical variables, analyzed by SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). The normality of the continuous variables was tested using the Kolmogorov–Smirnov test, and means were subsequently analyzed using either the two-tailed *t*-test (normal data distribution) or the Mann– Whitney *U*-test (skewed data) to compare two means where appropriate. Proportions were tested using the Chi-square test where appropriate. In all cases, statistical significance was established at $P < 0.05$.

RESULT

A total of 676 cycles were enrolled in this study. Demographic data and cycle characteristics of all patients are summarized in **Table 1**. There was no significant difference in the basal demographic conditions between the GH+ group and the GH- group, in terms of age (36.96 ± 4.77 vs. 36.84 ± 4.74, $P > 0.05$), basal FSH (9.42 ± 5.66 vs. 8.78 ± 3.87, $P > 0.05$), AMH (0.69 ± 0.31 vs. 0.68 ±0.32, $P > 0.05$), and AFC (5.54 ± 2.55 vs. 5.60 ± 2.47, $P > 0.05$). Both groups underwent a similar composition of conventional protocols ($P > 0.05$). Patients in the GH+ group had previously suffered more failed attempts (2.63 ± 1.81 vs. 2.28 ± 1.99, $P = 0.016$) but were inclined to have a lower dosage and shorter duration of Gn stimulation, in keeping with the consistently recognized beneficial effect of GH co-treatment from the earliest studies in the 1980's (31). Finally, the number of oocytes retrieved (3.64 ± 2.83 vs. 3.54 ± 2.80, $P > 0.05$) was equivalent, and further culture resulted in an equal number of 2PN oocytes (2.39 ± 2.27 vs. 2.32 ± 2.21, $P > 0.05$), number of transferrable embryos (1.90 ± 1.95 vs. 1.83 ± 1.86, $P > 0.05$) and number of good quality embryos (1.53 ± 1.70 vs. 1.42 ± 1.59, $P > 0.05$) between the two groups. There were more cycles in the GH+ group than in the GH- group performed frozen only due to suboptimal endometrial features or personal reasons (144 vs. 76, $P < 0.001$), thus, fewer cycles in the GH+ group than in the GH- group had fresh embryos transferred (87 vs. 153, $P < 0.001$). However, the cancelation of fresh embryo transfers due to abnormal fertilization, unfertilized or no transferrable embryos in both groups are similar (107 vs. 109, $P > 0.05$).

TABLE 1 | Demographic data and cycle characteristics of matched cycles with GH adjuvant ($n = 676$).

	GH+ group ($n = 338$)	GH- group ($n = 338$)	*P*-value
Age (year)	36.96 ± 4.77	36.84 ± 4.74	0.725
Infertility years	4.96 ± 3.80	4.79 ± 3.48	0.832
BMI	22.66 ± 2.85	22.58 ± 2.72	0.770
Basal FSH (IU/L)	9.42 ± 5.66	8.78 ± 3.87	0.356
AMH (ng/ml)	0.69 ± 0.31	0.68 ± 0.32	0.746
AFC (*n*)	5.54 ± 2.55	5.60 ± 2.47	0.725
Failed attempts	2.63 ± 1.81	2.28 ± 1.99	<0.001
Cycles conducted with Long Protocol	179	188	0.487
Cycles conducted with Antangonsis Protocol	159	150	0.487
Gn dosage (IU)	2358.28 ± 625.87	2474.19 ± 695.07	0.039
Gn duration (day)	9.81 ± 2.13	10.73 ± 1.99	<0.001
Number of oocytes	3.64 ± 2.83	3.54 ± 2.80	0.726
Number of 2PN (n)	2.39 ± 2.27	2.32 ± 2.21	0.796
Number of transferable embryos (n)	1.90 ± 1.95	1.83 ± 1.86	0.789
Number of good quality embryos (n)	1.53 ± 1.70	1.42 ± 1.59	0.493
No. of canceled cycle that frozen all because of endometrium or patients' require	144	76	<0.001
No. of canceled cycle resulted in unfertilized or abnormal fertilized	107	109	0.869
Number of cycles with fresh embryo transfered	87	153	<0.001

All values presented as mean ± SD.
P < 0.05 is considered statistically significant.
FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count; Gn gonadotropin; 2PN, 2 pronuclei.

TABLE 2 | Cycle characteristic and clinical outcomes of fresh embryo transferred cycles ($n = 240$).

	GH+ group ($n = 87$)	GH- group ($n = 153$)	*P*-value
Age (year)	36.25 ± 4.20	35.89 ± 4.87	0.538
Endometrial thickness (mm)	11.02 ± 2.07	11.22 ± 2.43	0.699
Number of embryo transferred	1.67 ± 0.47	1.77 ± 0.44	0.699
Number of transferred embryos on Day 3	78	135	0.738
Number of transferred embryos on Day 5/6	9	18	0.738
Good quality embryo rate in transferred embryos (%)	81.38% (118/145)	78.89% (213/270)	0.547
Biochemical pregnancy rate (%)	36.78% (32/87)	36.60% (56/153)	0.978
Clinical pregnancy rate (%)	35.63% (30/87)	32.68% (46/153)	0.479
Miscarriage rate (%)	13.33% (4/30)	41.30% (19/46)	0.009
Implantation rate (%)	23.45% (34/145)	19.26% (52/270)	0.607
Twin pregnancy rate per transfer cycle (%)	4.60% (4/87)	3.92% (6/153)	0.801
Ectopic pregnancy rate per transfer cycle (%)	1.15% (1/87)	2.61% (4/153)	0.656
Live delivery rate per transfer cycle (%)	29.89% (26/87)	17.65% (27/153)	0.028

All values presented as mean ± SD.
P < 0.05 is considered statistically significant.

In cycles that had fresh embryo transfer (data are shown in **Table 2**), there was no difference in demographic data, age, endometrial thickness, number of embryos transferred, proportion of embryonic development stage (cleavage/blastocyst), good quality embryo rate (81.38 vs. 78.89%, $P > 0.05$) or implantation rate (23.45 vs. 19.26%, $P > 0.05$). Surprisingly, the live birth rate per transfer cycle in the GH+ group was markedly higher than that of the GH- group (29.89 vs. 17.65%, $P = 0.028$), which might owe to significant lower miscarriage rate within the GH+ group (13.33 vs. 41.30%, $P = 0.009$). In order to further clarify whether the effect of GH on clinical results was related to patient age, we divided all the enrolled patients into POSEIDON group 3 (PG3, age<35 years old) or group 4 (PG4, age≥35 years old), and the results are presented in **Table 3**. All demographic data and clinical characteristics were comparable in the separate age groups. In PG3, younger patients were of equal age (31.35 ± 2.21 vs. 31.55 ± 2.27, $P > 0.05$), comparable basal FSH (9.73 ± 6.67 vs. 8.85 ± 3.80, $P > 0.05$), AMH (0.71 ± 0.31 vs. 0.69 ± 0.32, $P > 0.05$), and AFC (5.84 ± 2.97 vs. 6.15 ± 2.77, $P > 0.05$) between the GH+ group and GH- group. Patients in the GH+ group had a higher BMI than patients in the GH- group (22.01 ± 2.67 vs. 21.39 ± 2.57, $P = 0.033$). Further analysis of the difference according to the patients' BMI: lean (BMI<18.5 kg/m2); normal (18.5 kg/m2≤BMI<25 kg/m2); and overweight (BMI≥25 kg/m2), no significant difference in the subgroups distribution of patients was found between the two groups ($P > 0.05$). However, GH adjuvant showed a beneficial effect on the ovarian response, as the total Gn dosage was significantly lower (2351.39 ± 642.95 vs. 2577.80 ± 704.70, $P = 0.013$) with shorter Gn stimulation duration (9.69 ± 2.33 vs. 10.78 ± 1.95, $P < 0.001$) in GH+ group compared to GH- group while the composition of two protocols are similar between these groups ($P > 0.05$). Furthermore, 2019 ESHRE COS guideline has indicated that antagonist and agonist protocol are equally effective for poor responder, which could help us to better explain our results (28). A similar number of oocytes was retrieved (3.87 ± 2.76 vs. 4.40 ± 3.05, $P > 0.05$) and good quality embryos developed (1.42 ± 1.69 vs. 1.75 ± 1.61, $P > 0.05$), although the number of 2PN (2.39 ± 2.34 vs. 2.95 ± 2.39, $P = 0.025$) and transferrable embryos (1.79 ± 1.88 vs. 2.20 ± 1.87, $P = 0.034$) were fewer.

In the PG4 group, elderly patients were also of comparable age (39.60 ± 3.07 vs. 39.60 ± 3.04, $P > 0.05$) and equivalent ovarian reserve status between the GH+ and GH- groups, as the basal FSH (9.25 ± 5.07 vs. 8.75 ± 3.91, $P > 0.05$), AMH (0.68 ± 0.30

TABLE 3 | Demographic data and cycle characteristics of cycles with GH adjuvant in PG3 and PG4 ($n = 676$).

	PG3 (age < 35 years)			PG4 (age ≥ 35 years)		
	GH+ group ($n = 108$)	GH- group ($n = 116$)	*P*-value	GH+ group ($n = 230$)	GH- group ($n = 222$)	*P*-value
Age (year)	31.35 ± 2.21	31.55 ± 2.27	0.340	39.60 ± 3.07	39.60 ± 3.04	0.997
Infertility years	3.91 ± 2.58	4.23 ± 2.21	0.189	5.46 ± 4.16	5.08 ± 3.96	0.351
BMI	22.01 ± 2.67	21.39 ± 2.57	0.033	22.96 ± 2.89	23.19 ± 2.58	0.221
lean	9	7	0.635	9	2	0.114
normal	82	94	–	175	173	–
overweight	17	15	–	46	47	–
Basal FSH (IU/L)	9.73 ± 6.67	8.85 ± 3.80	0.756	9.25 ± 5.07	8.75 ± 3.91	0.427
AMH (ng/ml)	0.71 ± 0.31	0.69 ± 0.32	0.737	0.68 ± 0.30	0.67 ± 0.32	0.879
AFC (n)	5.84 ± 2.97	6.15 ± 2.77	0.264	5.40 ± 2.32	5.32 ± 2.25	0.653
Failed attempts	2.58 ± 1.79	1.83 ± 1.51	<0.001	2.65 ± 1.83	2.51 ± 2.17	0.041
Cycles conducted with Long Protocol	62	81	0.053	117	107	0.570
Cycles conducted with Antangonsis Protocol	46	35	0.053	113	115	0.570
Gn dosage (IU)	2351.39 ± 642.95	2577.80 ± 704.70	0.005	2361.52 ± 619.09	2422.05 ± 685.36	0.526
Gn duration (day)	9.69 ± 2.33	10.78 ± 1.95	<0.001	9.87 ± 2.03	10.71 ± 2.02	<0.001
Number of occytes	3.87 ± 2.76	4.40 ± 3.05	0.139	3.53 ± 2.86	3.09 ± 2.56	0.130
Number of 2PN (n)	2.39 ± 2.34	2.95 ± 2.39	0.025	2.39 ± 2.24	2.00 ± 2.03	0.036
Number of transferable embryos (n)	1.79 ± 1.88	2.20 ± 1.87	0.034	1.95 ± 1.98	1.64 ± 1.82	0.055
Number of good quality embryos (n)	1.42 ± 1.69	1.75 ± 1.61	0.053	1.58 ± 1.71	1.25 ± 1.55	0.018

All values presented as mean ± SD.

$P < 0.05$ is considered statistically significant.

FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; AFC, antral follicle count; Gn gonadotropin; 2PN, 2 pronuclei.

vs. 0.67 ± 0.32, $P > 0.05$), and AFC (5.40 ± 2.32 vs. 5.32 ± 2.25, $P > 0.05$) were well-matched. Similarly, the GH adjuvant improved the ovarian response by decreasing the duration of Gn stimulation (9.87 ± 2.03 vs. 10.71 ± 2.02, $P < 0.001$) with similar protocols. The number of oocytes retrieved (3.53 ± 2.86 vs. 3.09 ± 2.56, $P > 0.05$) was equal between the two groups. Further culture with a parallel number of oocytes, showed a significant increase in the number of good-quality embryos (1.58 ± 1.71 vs. 1.25 ± 1.55, $P = 0.032$), indicating that the oocyte utilization rate was greatly increased with improved embryo quality.

Clinical outcomes are further analyzed as well in **Table 4**. No matter whether in the GH+ group or the GH- group, there were more patients in PG4 compared with PG3, which is in line with the realistic incidence of ovarian reserve decline being higher in elderly patients. In both PG3 and PG4, there was no difference in age ($P > 0.05$ in PG3 and $P > 0.05$ in PG4), endometrial thickness ($P > 0.05$ in PG3 and $P > 0.05$ in PG4), number of embryo transferred ($P > 0.05$ in PG3 and $P > 0.05$ in PG4), embryonic development, and the proportion of good quality embryo transferred ($P > 0.05$ in PG3 and $P > 0.05$ in PG4) between the GH+ and GH- groups. In PG3 and PG4, clinical pregnancy rate, implantation rate, twin pregnancy rate, and ectopic pregnancy rate were equal between the GH+ group and the GH- group. Although GH did not reveal any beneficial in terms of biochemical pregnancy rate, GH supplement in PG4 achieved a borderline improved clinical pregnancy rate (36.7 vs. 23.0%, $P = 0.071$) and a significant increase in live birth rate (27.3 vs. 9.2%, $P = 0.003$), accompanied with decrease in miscarriage rate significantly (18.2 vs. 60.0%, $P = 0.005$).

DISCUSSION

In this retrospective study based on POSEIDON criteria, poor ovarian reserve patients were enrolled and further classified as PG3 and PG4. The results revealed that GH adjuvant during COH benefits the ovarian response, and promotes the clinical outcome of patients over 35 years old who underwent IVF/ICSI treatment. These results were similar to our previous self-controlled research, but demonstrated a more specific subgroup of patients (9). In a study by Cochrane, GH administration helped improve the live birth rate of PORs, but the research did not define the subgroups of POR patients who actually benefited from the GH adjuvant (32). Before the POSEIDON criteria were posed, the Bologna criteria were once popular. However, the Bologna criteria have a fuzzy definition of the threshold of ovarian reserve markers (i.e., AFC <5–7 follicles or AMH <0.5–1.1 ng/mL) and of "other cause in POR" (26). In addition, it brings the risk of categorizing patients with significant differences in biological characteristics (33). Interestingly, our studies were conducted only during the time of diagnostic criteria reform. In our previous study, we enrolled patients with POR diagnosed by the Bologna criteria. Despite the heterogeneity of patients, we conducted it with a self-control design to minimize the heterogeneity, resulting in a significant positive conclusion (9). Along with the progress in POR criteria, we carried out this study with the aim specifying the subgroup in which GH could be the most beneficial. We enrolled patients who complied with the definition of PG3 and PG4 (AMH<1.2 ng/ml), avoiding interobserver differences in AFC.

TABLE 4 | Cycle characteristic and clinical outcomes of fresh embryo transferred cycles in PG3 and PG4 ($n = 240$).

	PG3(age < 35 years)			PG4 (age ≥ 35 years)		
	GH+ group ($n = 27$)	GH- group ($n = 66$)	*P*-value	GH+ group ($n = 60$)	GH- group ($n = 87$)	*P*-value
Age (year)	31.30 ± 1.75	31.33 ± 2.38	0.569	38.48 ± 2.85	39.35 ± 3.11	0.095
Endometrial thickness (mm)	11.33 ± 1.80	11.96 ± 2.11	0.155	10.82 ± 2.23	10.70 ± 2.48	0.443
Number of embryos transferred	1.74 ± 0.45	1.83 ± 0.38	0.308	1.63 ± 0.49	1.71 ± 0.48	0.345
Number of transferred embryos on Day 3	26	61	0.668	52	74	0.784
Number of transferred embryos on Day 5/6	1	5	0.668	8	13	0.784
Good quality embryo rate in transferred embryos (%)	78.7% (37/47)	80.2% (97/121)	0.833	82.7% (81/98)	73.8% (110/149)	0.105
Biochemical pregnancy rate (%)	29.6% (8/27)	51.5% (34/66)	0.054	40.0% (24/60)	25.3% (22/87)	0.059
Clinical pregnancy rate (%)	29.6% (8/27)	39.4% (26/66)	0.375	36.7% (22/60)	23.0% (20/87)	0.071
Miscarriage rate (%)	0% (0/8)	26.9% (7/26)	0.100	18.2% (4/22)	60.0% (12/20)	0.005
Implantation rate (%)	76.9% (10/47)	24.0% (29/121)	0.711	24.5% (24/98)	15.4% (23/149)	0.076
Twin pregnancy rate per transfer cycle (%)	7.4% (2/27)	4.5% (3/66)	0.626	3.0% (2/66)	3.4% (3/87)	1.000
Ectopic pregnancy rate per transfer cycle (%)	0.0% (0/27)	6.1% (4/66)	0.319	1.5% (1/66)	0.0% (0/87)	0.431
Live delivery rate per transfer cycle (%)	29.6% (8/27)	28.8% (19/66)	0.935	27.3% (18/66)	9.2% (8/87)	0.003

All values presented as mean ± SD.
$P < 0.05$ is considered statistically significant.
No, Number.

Among all enrolled patients with poor ovarian reserve, lower dosages and shorter durations of Gn stimulation were detected in the GH+ group of all POR patients, which implies an enhancement in ovarian response, illustrating the important role of GH in the proliferation and differentiation of granulosa cells, as demonstrated in animal research (14). In further analysis, GH addition promoted the number of good quality embryos, clinical pregnancy rate, and live birth rate in women with poor ovarian reserves who were older than 35 years. Importantly, encouraging results in the GH+ group also echoed our previous work (9), the reported data by Yovich et al. (8, 34) and data revealed by Tesarik et al. (35) in a RCT. The homogeneity of patients was better in our research given that Yovich et al. enrolled patients diagnosed with broad Bologna criteria and Tesarik et al. recruited patients with a smaller range of ages. Although a recent multicentric randomized placebo-controlled trial, published by Norman et al. (18), provided no evidence for an increase in the live birth rate from the large dosage GH co-treatment and not recommended for widespread use in PORs, the patients enrolled were in a broader criteria in that the study was designed much earlier than the newly classification proposed. Furthermore, that study was limited by recruitment failure, a feature acknowledged by the authors. However, they also believed that the currently recognized definition such as POSEIDON may have unmasked a subgroup of PORs that can really benefit from GH. Though the live birth is multifactorial, the quantity and quality of oocytes equally contribute to pregnancy outcomes in women with POR and age is the only predictor of quality available (36). As ovarian reserve is irreversible, GH addition may increase oocyte quality as well as ovarian response, especially in aged patients with poor ovarian reserve, thus increasing the live birth rate of these patients (37).

Patients younger than 35 years old in the GH+ group had higher BMI compared to patients in the GH- group, however, we reanalyzed the difference according to the patients' BMI and found no significant difference in the subgroup distribution of patients between the three groups ($P > 0.05$). They were treated with significantly lower total Gn dosages and shorter Gn stimulation durations. This economic effect of reducing the total Gn dosage and duration was shown among all enrolled patients, which implies that GH adjuvant promoted the ovarian response, which is a different conclusion compared with our previous study (9). Besides, Ahmed et al. has reported that there was no significant difference among poor responders with different BMI in gonadotropin dose, duration of stimulation, number of oocytes retrieved, number of embryos, transferred embryos in a prospective cohort study (38), which may provide indirect evidence that GH administration improve ovarian response in patients younger than 35 years old. Although a comparable number of oocytes was collected, fewer 2PN and transferrable embryos were formed. However, this difference failed to reach statistical significance in number of good quality embryos, causing us to reconsider the value of GH in young patients. Another study focusing on young patients is urgently needed.

Development in laboratory research in older women provides a theoretical basis for our study. Mitochondria are considered to be a keystone of oocyte development potential, but both the quality and the quantity of mitochondria and mtDNA number in oocytes are significantly decreased with female aging, and the addition of GH could partially amend these features (13). It has recently been reported that GH co-treatment in older patients with reduced ovarian reserve can modulate the density of GH receptors in granulosa cells and further improve clinical outcome (39).

This study has its limitation as a retrospective analysis, but it still provides important clues aiming to improve therapeutic intervention strategies for POR patients. It is the first paper based on the POSEIDON criteria to distinguish specific subgroups of POR that GH works effectively, which may clarify the detailed adjuvant methods and specific subgroups patients of

GH treatment and avoiding extra economic burden for patients who are invalid. Molecular marker detection is still required to further support our results in a well-designed, multicenter, prospective RCT.

Taken together, 2 IU of GH adjuvant ∼6 weeks preceding OPU is sufficient to reveal the beneficial effects of GH on promoting the live birth rate for PG4 patients diagnosed by POSEIDON criteria.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by 2016ZSLYEC-061. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XY conceived and designed the study. MC made substantial contributions to data analysis and interpretation. LG and YW completed the data collection. XY, XL, and CF reviewed the content and critically revised the manuscript. All authors contributed to writing the manuscript.

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REVIEW
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The Evolving Concept of Poor-Prognosis for Women Undertaking IVF and the Notion of Growth Hormone as an Adjuvant; A Single-Center Viewpoint

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IVF is currently regarded as a successful new technology with the number of IVF children currently well over 8 million worldwide. This has been achieved by an explosive plethora of facilities. However, from its earliest history, IVF has been beset by poor-prognosis on a treatment cycle basis, an aspect which has been a constant feature for the majority of treatments to this stage. The 2019 Australian and New Zealand Assisted Reproduction Database (ANZARD) report shows that IVF clinics have live birth productivity rates (from combined initiated fresh and frozen cycles) ranging from 9.3 to 33.2%. Over the past 40 years there have been a number of innovations which have steadily moved the success rates forward, but progress is held back by an intransigent group of women who can be classified as being poor-prognosis from one or more adverse factors, namely advanced age (>40 years), poor ovarian response (POR) to ovarian stimulation, inability to generate high quality blastocyst-stage embryos, recurrent implantation failure, or recurrent early pregnancy losses. A number of strategies are variously applied including the use of recombinant growth hormone (GH) adjuvant therapy. Our retrospective studies at PIVET over the past decade show a 6.2-fold chance of live birth for fresh cycle embryo transfers following GH injections of 1–1.5 IU daily given for 3–6 weeks in the lead-up to the trigger for ovum pick-up. We have also recently reported the live birth rates from frozen embryo transfers utilizing those blastocyst embryos generated under GH influence and showed the live birth rate was 2.7-fold higher in a carefully matched poor-prognosis group. This experience has been compared to the total 42 GH studies reported since the year 2000, the majority matching those of PIVET with significant increases in both oocyte and embryo utilization rates but only ∼50% are followed by elevated live birth rates. We argue that this discrepancy relates to failure in addressing other causes of poor-prognosis along with the wastage of transferring more than a single embryo in the fresh cycle, when ANZARD data indicates a significantly higher chance of live birth from frozen embryo transfers.

Keywords: poor-prognosis, IVF adjuvants, poor ovarian responder (POR), growth hormone (GH), adult growth hormone deficiency (AGHD), oocyte utilization rate, embryo utilization rate, live birth productivity rate

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INTRODUCTION

The concept of poor-prognosis for women undertaking *in-vitro* fertilization (IVF) is embedded in the early history of human IVF and continues to be a stubborn but evolving concept. One attempt to define the poor-prognosis woman focussed on those with low ovarian reserve who therefore had limited response to ovarian stimulation strategies, even applying maximal dosage of gonadotrophins. The ESHRE working group (1) categorized a poor ovarian responder (POR), applying a definition where women had at least two of the following three features:

(i) Advanced maternal age (≥40 years) or any other risk factor for POR;
(ii) A previous POR (≤3 oocytes with a conventional stimulation protocol);
(iii) An abnormal ovarian reserve test (ORT) i.e., antral follicle count (AFC), <5–7 follicles or anti-Mullerian hormone (AMH) level <0.5–1.1 ng/ml (For SI units; <3.5–8 pmol/L).

Added point: The ESHRE working group accepted that two episodes of POR after maximal stimulation are sufficient to define a patient as poor responder in the absence of advanced maternal age or abnormal ORT.

"By definition, the term POR refers to the ovarian response and, therefore, one stimulated cycle is considered essential for the diagnosis of POR. However, patients over 40 years of age with an abnormal ORT may be classified as poor responders since both advanced age and an abnormal ORT may indicate reduced ovarian reserve and act as a surrogate of ovarian stimulation cycle. In this case, the patients should be more properly defined as expected PORs."

POOR-PROGNOSIS VS. POR

The aforementioned Bologna definition has been criticized from the outset, mainly because it fails to address the question of oocyte quality and the relevance of risk factors which, together influence embryo quality. Furthermore, standardization of both AFC and AMH assays remains problematic (2). The Israeli group of Younis and colleagues indicated 6 main areas of debate but our view would contend that POR is but one factor under a broader problem of poor-prognosis factors limiting the chance of generating live births from IVF.

To understand poor-prognosis requires a review of the historical evolution of IVF to its current improved, but still rather imperfect, position. Furthermore, when attempting to evaluate adjuvant therapies given to women who had experienced repeated failures in IVF programmes, we faced several problems related to modern IVF evolutionary factors, namely the increased reliance on cryopreserved embryos, the progress toward single embryo transfers (preferentially undertaken at the blastocyst stage) and the methodology of evaluating embryo quality. Along with those evolutionary trends, "advanced ovarian stimulation protocols" have also emerged. For comparative evaluations of these trends new definitions have been introduced such as a Productivity Rate (3), meaning the total number of live births arising from a single IVF cycle initiated. The Productivity Rate may be classified according to a particular clinical regimen (±adjuvant therapy), or a modified laboratory protocol. Ideally, the Productivity Rate reflects the real outcome, but clinical studies are often frustrated because of an increasing trend to cryopreserve all embryos (so called "freeze-all" protocol) and embryos may remain in cryopreservation for several years, unable to be evaluated during a particular study period. Other frustrations for research studies include the 10-month long period from IVF cycle initiation (e.g., Day of commencing ovarian cycle tracking or stimulation) to birth outcome. Surrogate measures over a shorter period may prove to be valid, such as the oocyte utilization number/or rate (being the number/or proportion of oocytes which result in embryos which prove suitable for fresh-cycle transfer or cryopreservation, ideally at blastocyst-stage). Oocyte Utilization can be rated per total oocytes recovered at oocyte pick-up (OPU) or per number of 2PN-stage oocytes resulting after fertilization (such oocytes reflecting a "mature" group). This latter category may reasonably be termed Embryo Utilization Rate (3). These terms were introduced during PIVET's earliest GH-adjuvant studies (4) and have proven useful, essentially validated, in subsequent studies and reports (5, 6). Some reports use the term cumulative live birth rate (CLBR) but that term historically related to several OPU cycles, hence a specific term such as productivity rate should be preferable (3). Terminology aside, the concept is now incorporated into the annual Australian and New Zealand Assisted Reproduction Database (ANZARD) report (7) which also reveals that "freeze-all" was conducted in 24.2% of the 47,545 autologous fresh IVF cycles undertaken in 2017.

At PIVET the term poor-prognosis has been applied according to any one of 5 criteria, namely:

(i) All women aged 40 years and above
(ii) All women categorized as poor-prognosis from previous IVF, meaning repeated failures (≥3) RIF
(iii) All PORs (generating ≤4 oocytes despite FSH dosing maximized at 450 IU daily)
(iv) All cases with "E" categorization according to PIVET FSH-dosing algorithms [AMH <5 pmol/l & AFC <5 follicles; (5)] matching ORT according to Bologna criteria
(v) All cases where resultant embryo quality rated poor, meaning no suitable blastocysts for cryopreservation (Good prognosis in IVF generates 8–12 oocytes resulting in ≥3 blastocysts with gradings 3BB or better).

For the purpose of this article, it can be seen that historically the diagnosis of poor-prognosis is defined after one or more IVF attempts have already been undertaken, an expensive and unhappy scenario for those patients who have failed to achieve a pregnancy and ensuing live birth. Ideally, the diagnosis should be established following primary assessment of the infertile couple, so that remedial strategies can be introduced from the outset. One of those strategies can be the application of adjuvants such as growth hormone for which the notion of adult growth hormone deficiency (AGHD) has been proposed (8).

Historical Perspective

From the very beginning of human IVF, poor-prognosis has been intrinsic to the treatment mode. This concept of poor-prognosis has continued to change with the evolution of methodologies and technologies in IVF. The 40th birthday of the world's first IVF live birth, Louise Brown in July, 2018 was lauded world-wide as she is now accompanied by more than 8 million IVF offspring. However, it is relevant for this discussion concerning poor-prognosis in IVF, to note that her birth followed a decade of effort by the acknowledged "Fathers of IVF" whereby 282 couples underwent 457 cycles of treatment and 112 women completed an embryo transfer procedure (9). Gynecologist Patrick Steptoe, working in Oldham near Manchester UK, undertook the vast majority of oocyte pick-ups (OPUs) by laparoscopy, a novel procedure he introduced to Britain in 1967 after his training in France with Raoul Palmer, the pioneer of modern laparoscopy. There were some women who required laparotomy due to dense pelvic adhesions precluding safe access to the pelvis via laparoscopy. Physiologist Robert Edwards, from Cambridge developed the IVF protocols and performed the embryology procedures with technical assistance from nurse Jean Purdy, who later qualified as an embryologist. After many years of frustration, including several biochemical pregnancies and an ectopic pregnancy in 1975, Steptoe advised removal of, or preliminary clipping of the fallopian tubes. Around the same time, Edwards encouraged the switch from ovarian stimulation, which had attendant problems with the luteal phase, to tracking of the natural cycle. This was facilitated by introducing the very sensitive HiGonavis pregnancy kit from Mochida Pharmaceuticals which could detect both hCG and LH levels as low as 20 IU/L in urine. The final phase of their work was performed in nearby Dr. Kershaw's Cottage hospital where 79 couples were admitted and 68 women reached the stage of laparoscopy with 55 achieving successful OPU. However, only 32 cases had a Day-2 embryo suitable for transfer; of these, 4 clinical pregnancies ensued—one miscarried at 11 weeks, a second delivered pre-term at 21 weeks with neonatal demise soon after the birth. This case was categorized as a post-amniocentesis loss and caused the "Fathers" to advise against routine testing in the future. The 3rd pregnancy resulted in the delivery of Louise Brown on 25 July 1978 and the 4th resulted in the delivery of Alistair MacDonald on 14 January 1979 (9).

This historical detail is relevant for this article as 3 livebirths from 457 cycles initiated (<1%) or from 112 embryo transfers (3%) can be considered poor-prognosis by current standards. The Kershaw history was somewhat better as the pregnancy rate was 5% of the 79 women admitted (initiated), 6% of the 68 laparoscopies, 7% of OPUs, and 12.5% of ETs. Progressing beyond the perinatal phase, and depending on definition for livebirth dating being either 20 or 28 weeks, the live birth rate was 2 for the 79 women initiated (2.5%). These results have stimulated a publication from social scientists praising the "Mothers of IVF" and honoring patient 38 who endured 10 laparoscopies, achieving only an ectopic pregnancy for all her compliant efforts. Actually 11 women had 5 or more OPUs and deserve honorable mentions (10). In fact, the mothers of the two pregnancies that continued to surviving live births can be classified as "good prognosis" as their pregnancies resulted from a single laparoscopy. This implies that the other 280 women had endured a poor-prognosis (99.3%).

REDUCING THE POOR-PROGNOSIS FEATURE 1978–1982

Whilst IVF practitioners in modern day might dismiss the aforementioned history as "teething problems," it is also relevant to point out that the ensuing 4 years were also difficult with only 9 pioneering groups worldwide (**Table 1**) reporting livebirths to July 1982 and a dozen by end 1982 (9). Of interest, almost all these units followed the Edwards dictum of pursuing natural cycles, but units in only 3 countries achieved livebirths from that protocol (4 units; Oldham UK; Royal Women's Hospital unit in Melbourne, Australia; the Clamart unit in Paris, France; and the unit at Sèvres, also in Paris, France. The Frydman unit at Clamart had developed a rapid plasma radio-immunoassay for LH as an advance over the HiGonavis test). All the others, as well as these 4 units eventually, abandoned Natural Cycle IVF for various forms of ovarian stimulation in IVF. In fact, the pioneering unit with the second successful live birth—the unit from from Kolkata (Calcutta), India with the birth of Kanuprija "Durga" Agarwal on 3 October 1978, applied ovarian stimulation (HMG) and other techniques which were at least 30 years in advance of then current IVF practice. These included the idea of embryo cryopreservation and subsequent frozen embryo transfer (FET) in a natural cycle. Of the other pioneer units shown in **Table 1**, eight used Clomid with HCG trigger and three applied HMG with HCG trigger. Only two units applied any luteal phase support, that being progesterone injections in the Norfolk, USA unit and the progestogen medroxyprogesterone acetate (MPA) in the Perth, Australia unit. Five of the pioneer centers reported twin pregnancies, three with livebirths in 1982 and two others with livebirths in early 1983. One of the latter was the first report of monozygotic twins from IVF.

REDUCING THE PROBLEM OF POOR-PROGNOSIS 1983–2003

In the two decades from 1983, IVF outcomes improved, meaning that livebirths were being reported from all over the world. Although pregnancies tended to be sporadic for start-up units, the live birth rates per initiated cycle for established facilities were rarely better than 10%. That decade was notorious for publications reporting variously irregular numerator and denominator criteria to provide the "best look." Many units reported on favorable segments of practice. Therefore, it was generally not possible to know accurately whether IVF methodology was improving or whether the rising number of IVF babies was simply the result of IVF units selecting the younger, easier cases suited to the early protocols and laboratory methods (11). In fact, a cynical view might be that many newer start-up units were overstimulating young women who had highly responsive ovaries to boost positive outcomes. This period saw numerous cases of ovarian hyperstimulation syndrome

TABLE 1 | Lists the pioneer IVF centers which established livebirths from IVF, beginning with Louise Brown in July 1978 and documenting 9 successful centers to her 4th birthday (July 1982); thereafter another 3 centers to the end of 1982.

Team	Country	City	Main members	First L/Birth	Ov Stim/Tr/LS[#]
1	Britain	Oldham, Manchester, UK	Steptoe, Edwards, Purdy	Jul-78 Jan-79	Nil/nil/nil[$]
2	India	Kolkata/Calcutta, India	Mucherjee, Muckhergee, Battacharya	Oct-78	hMG/hCG/FET
3a	Australia	Melbourne, Australia	Wood, Johnston, Lopata	Jun-80	Nil/nil/nil
3b	Australia	Melbourne, Australia*	Wood, Leeton, Trounson	May-81 Jun-81	Clomid/hCG/nil 9 L/Bs, 10 infants, Twins
4	USA	Norfolk, Virginia, USA*	Jones, Seeger-Jones, Garcia, Acosta, Veek	Dec-81 Mar-83	hMG/hCG/P4 Inj Twins
5a	France	Clamart, Paris, France	Frydman, Testart, Lasalle, Papeirnik	Feb-82	Nil/nil/nil
5b	France	Sèvres, Paris, France	Cohen, Plachot, Mandelbaum	Jun-82	Nil/nil/nil
6	Britain	London, UK*	Craft, Yovich, Green, Shelton, Bernard	Apr-82	Clomid/hCG/nil Twins
7	Germany	Erlangen, Bavaria, Germany	Trotnow, Kniewald, Habermann	Apr-82	Clomid/hCG/nil
8	USA	Los Angeles, California, USA	Marrs, March, Mishell	Jun-82	Clomid/hCG/ P4 inj
9	Australia	Perth, Western Australia*	Yovich, Pusey, De Atta, Roberts, Reid, Grauaug	Jul-82 May-83	Clomid/hCG/MPA Twins (MZ)
10	Sweden	Stockholm, Sweden	Hamberger, Nilsson, Wikland, Enk	Sep-82	Clomid/hCG/nil
11	Israel	Sheba, Ramat Gan, Israel	Maschiach, Dor, Ben-Rafael	Sep-82	Clomid/hCG/P4 inj
12	Austria	Vienna, Austria*	Feichtinger, Kemeter, Szalay	Oct-82 Nov-82	Clomid/hCG/nil Twins

References for the data in this table can be found in Yovich and Craft (9). These first IVF livebirths were "hard-won" with Team 3b generating the best results; from 115 initiated cycles 14 pregnancies (12.2%) and 9 deliveries ensued (7.8% live birth rate). [#]*Ov stim/Tr/LS, Ovarian stimulation/Trigger/Luteal support.* [$]*nil/nil/nil, no ovarian stimulation/no trigger injection/no luteal support. P4 Inj, progesterone injections; MZ, monozygotic.*

**The first IVF twin live births—in order 1. Melbourne, Australia; 2. London, UK; 3. Vienna, Austria; 4. Norfolk, Virginia, USA; and 5. Perth, Australia.*

(OHSS) and rising rates of high-order multiple pregnancies; the case of "Octomum" being the most notorious with 14 babies (4 singletons, 1 twin, and 1 octuplet) in one woman arising from a single initiated IVF cycle with ET and subsequent FET procedures (12). However, a true technical advance in IVF during this period was the introduction of ICSI in the early nineties (13), resulting in a solution for most male-factor causes of infertility and the potential avoidance of unexplained complete failed-fertilization (14). This advance broadened the indications for IVF and enabled the successful management of even azoospermic males when applied in concert with Vasal flush, PESA, MESA, and micro-TESE procedures (15, 16).

During this decade there were other definable progressive advances to IVF methodology which had the effect of further expanding the spectrum of infertility case scenarios which were responsive. In particular the introduction of gonadotrophin releasing hormone analogs, initially agonists (GnRHa) from the mid-1980's and later antagonists (GnRHant) in the mid-2000's. These introductions created control over the ovulation process, reducing elevated LH levels and preventing premature LH surges. This also enabled optimization of the ovulation trigger whereby ovulation could be delayed, to be triggered once maturation [based on ovarian follicle dimensions on pelvic ultrasound and serum estradiol (E2) levels] had been reached. The trigger injection included the use of GnRHa to replace HCG in cases managed with GnRHant who had high follicle numbers, with consequent near complete avoidance of OHSS (17).

In response to the several problems of OHSS, multiple pregnancies (with the associated problem of pre-term deliveries) and the complaints of high failure rates these two decades saw the increasing regulation of IVF practices. This was mostly self-regulatory by guidelines advised through societies such as the American Society of Reproductive Medicine (ASRM), the European Society of Human Reproduction and Embryology (ESHRE), the Fertility Society of Australia (FSA), and similar societies in most countries. In some countries there was a perceived need to introduce legislation resulting in statutory controls, such as the Human Fertility Embryology Act (1990) in the UK and similar Acts in some states of Australia. However, this type of regulation is now perceived to be unnecessary (18). A strong emphasis is now placed on teaching and training leading to improved laboratory methods which included the use of commercially prepared refined culture media, adapted for specific purposes i.e., flushing media, fertilization media, cleavage stage media, and blastocyst culture media (19). Specific cryopreservation media have also been developed for the advanced vitrification technique introduced from 2007 (20).

EVOLVING CONCEPT OF POOR-PROGNOSIS FROM 2004 TO CURRENT

Over the aforementioned historical period, the concept of "poor-prognosis" was largely changed by the development of IVF

methodologies to ensure a high-quality clinical service in the setting of "tight" laboratory processes and continuous data evaluation enabling IVF clinics to rate their performance and be rated by independent assessment. In Australia and New Zealand this is enacted by an annual accreditation process by the Reproductive Technology Accreditation Committee (RTAC) acting under the auspices of the FSA. This self-regulatory system is strengthened by the requirement of accreditation so that patients attending the IVF unit can be eligible to receive the substantial Medicare benefits provided by the National Governments of Australia and New Zealand.

The improvement in cryotechnology has led to the consideration of a new treatment concept i.e., the segmentation of IVF treatment, with embryo transfer performed in subsequent frozen embryo transfer (FET) cycles. In practical terms this may result in a "freeze-all" cycle which commits all embryos (best at the blastocyst stage) to cryopreservation by Vitrification, and which is best applied using the Cryotop method (20). Whilst the idea of routine segmentation for all is not yet considered to be the best approach, many clinics in the Australian setting are currently committing their best blastocysts to the freezer, transferring the second tier in the fresh cycle. This approach is gaining popularity, contingent upon the data outcomes reported in the Australian and New Zealand Assisted Reproduction Database (ANZARD) (7) which reveals a higher live birth rate from FET cycles than fresh ETs (**Table 2**). However, despite all the aforementioned progress, ANZARD reports that the results of IVF across the 91 Fertility Clinics operating during 2017 in Australia ($n = 83$) and New Zealand ($n = 8$) vary widely. The productivity rate (live births per cycle initiated; fresh and frozen autologous) ranged from 9.3 to 33.2% for data covering 98% of clinics; i.e., all those 88 of 91 clinics undertaking >50 OPU cycles (7). The report provides no analysis for the wide range and data concerning adjuvants is not collected at this stage.

TABLE 2 | Live birth outcomes from all fresh and frozen embryos transferred in Australia and New Zealand 2017.

Parameter	Autologous cycles with fresh transfer (all ages)	Autologous thaw cycles (all ages)
Initiated cycles	47,545	29,808
Cycles with OPU	42,632	–
Freeze-all cycles	12,110	–
Embryo transfer cycles	24,095	28,770
Clinical pregnancies	7,529	10,379
Live deliveries	5,803	8,310
Live deliveries per initiated cycle[#]	12.2%	27.9%*
Live deliveries per initiated cycle (excluding freeze-all cycles)	16.4%	–
Live deliveries per embryo transfer cycle	24.1%	28.9%*
Live deliveries per clinical pregnancy	77.1%	80.1%*

Data extracted from ANZARD report [(7); Table 9; fresh IVF ± ICSI cycles and Table 13; FET cycles]. Shows that the live birth rates per initiated cycle as well as per embryo transfer procedures, are significantly higher for frozen embryo transfers; 89.4% single embryo transfers; 82.0% blastocyst-stage transfers, 91.5% being cryopreserved by vitrification. The live deliveries (births) per initiated cycle may be compared to the outcomes reported in ***Table 1*** *over the years 1978–1982, where the best from the first 12 IVF Teams was 7.8%; not much lower than the fresh cycle outcome of 12.2 and 16.4% shown here. Within this ANZARD report, Figure 1 shows the live birth delivery per initiated fresh (excluding freeze-all) and thaw autologous and recipient cycle among 88 of the 91 fertility clinics (i.e., those performing >50 OPU cycles for the year) ranged from a low of 9.3% to a high of 33.2% across all ages. Information about clinics use of adjuvants and add-ons is not available in the report but we are aware that GH was used in 22% of OPU cycles in one of the IVF centers reporting live births >30% of initiated cycles across all ages. [#]At least one live infant at delivery; singletons 96.8%. *p < 0.0001 Chi-square with Yates correction.*

CHANGING CLINICAL PROFILES OF COUPLES UNDERTAKING IVF

Historically IVF was applied for mainly underlying female factors classified as tubal, endometriosis, and other pelvic disorders; but increasingly clinics have added ovulation disorders and male factor infertility; the former to avoid multiple pregnancies arising from ovarian stimulation and the latter because of the introduction of ICSI. Nowadays, ANZARD (7) shows that 10% of cases are designated combined male and female factors, but the highest infertility categorization is "unexplained," being more than 50% of cases. This phenomenon has led to some critical articles in the literature implying that IVF is being applied to many cases with inadequate workup; cases which might result in spontaneous pregnancies if managed better. One approach which increases the chances of avoiding IVF by careful workup, close monitoring and applying an Assessment Cycle has been published recently (21). Such a comprehensive approach identifies cases which can benefit by attention to nutritional health factors, coital timing, by offering oral therapies for disordered ovulation, by tubal flushing with lipiodol, by intra-uterine insemination (IUI) for negative post-coital tests and by hormonal supports where indicated during the luteal phase and early pregnancy. However, the most important factor for conception is female age, hence the argument about non-IVF treatments may hinge on available opportunity, such being greatest for young women <35 years and least for women ≥40 years.

OVARIAN STIMULATION SCHEDULES INFLUENCING POOR-PROGNOSIS

Further criticism of the Bologna criteria for POR, concerns the definitions of a standard and maximal stimulation schedule which, in the ESHRE context, means 150–225 IU rFSH. A more advanced dosage algorithm enabling a wider dosage range, targeted to multiple patient characteristics has been proposed (22) and subsequently validated by a prospective study within the same IVF unit (5). Such a targeted algorithm optimizes oocyte recovery to 10 ± 2 oocytes across the range of AFC and AMH categories and can improve the chance of live births, even in older women, with the effect of reducing the proportion of women labeled as poor-prognosis. A further, recently introduced, novel algorithm described as the POSEIDON stratification of low prognosis patients was recently proposed (23) and data is already beginning to appear which tends to validate its utility (24).

However, a confounder in the historical story of ovarian stimulation for IVF is the idea of minimal stimulation regimens which emerged during the 1990's when cases of OHSS were relatively common and included some reports of mortality. The idea was strongly promoted by clinics in Japan which reported "favorable" pregnancy rates per embryo transfer, without experiencing OHSS over 20 years, but not really disclosing the full story. However, the reality has been finally revealed by the Ethics Committee of the Japan Society of Obstetrics and Gynecology (25). Chairman Professor Saito shows that Japan has actually experienced the lowest live birth rate per initiated fresh IVF cycle reported anywhere in the world, the rate being 4.13% of OPUs undertaken in 2015. Furthermore, the productivity rate combining FET cycles is extremely low as the number of women having supernumerary embryos frozen (after fresh ET) is a very low proportion of the total initiated cycles as the majority have 0–4 oocytes recovered. These results can be contrasted with the ANZARD report which shows that in 2017, the SET rate in Australia and New Zealand was 89.4% and multiple pregnancies were a low 3.6%, being twins only without a single high-order live birth. This ensured that 80.2% of IVF infants were full-term singletons with normal birthweight. These favorable trends were achieved without any reduction in the live delivery rates, which actually increased to 26.8% per embryo transfer procedure (a significant rise from 22.5% over the 5-year period from 2012). There was a marked increase in the proportion of FET livebirths rising to 54.1% from 41.9% over the 5-year period with FET livebirth rates being 28.9% compared with 24.1% per ET for the fresh cycles. The markedly better FET implantation rates are shown in **Table 2**. The proportion of women undertaking IVF in the three age categories was similar being 36.6% for under 35 years; 36.2% for those aged 35–39 years; and 27.2% for those aged 40 years and over. Livebirth outcomes per initiated autologous cycle for those respective age ranges were 18.6% fresh and 32.0% frozen; 12.0% fresh and 28.0% frozen; 3.9% fresh and 17.3% frozen. Clearly, FET cycles generate more live births, a finding which is most marked with advanced female age. Furthermore, those pregnancies arising from FET cycles have a significantly higher chance of progressing to livebirths (80.1 vs. 77.1%; $p < 0.001$).

ADJUVANTS IN IVF

Notwithstanding the aforementioned progress in IVF methodologies, at least 10 categorical areas can be identified for improvement (**Table 3**). Many interventions (adjuvants, adjustments, and add-ons) have been introduced into the basic IVF model with a view to improving the chance of achieving a live birth from each cycle initiated, as listed in the table. These have included adjustments to improve ovarian responsiveness and the ovulatory response (to trigger), adjuvants to improve oocyte maturation, adjuvants to improve both implantation and placentation thereby diminishing pregnancy losses as well as add-ons for early pregnancy supports. The focus of this presentation is that of one adjuvant, namely GH. Although several of the add-ons listed in **Table 3** are widely used, none have reached universal acceptance from the perspective of Cochrane (26, 27) or NICE (28) and their use has drawn rather scathing criticism (29, 30) because of the additional costs for unproven benefit.

The historical preamble in this article was provided to show a poor-prognosis group has been evident in IVF from the beginning and that most of the useful developments for IVF to current day have been dependent on improving and tightening the protocols introduced in the early years. These can be summarized as focussing on the ovarian stimulation schedule and the trigger with a view to generating around 10 oocytes per IVF cycle thereby minimizing any risks to the woman. The translation of those 10 oocytes in best units is currently 1 good quality blastocyst-stage embryo transferred in the fresh cycle and an average of 2 blastocysts reaching sufficiently high grading to be vitrified for future FET attempts. This 30% oocyte utilization rate reflects current limited knowledge concerning oocyte maturation and controlling the age-dependent rate of chromosomal segregation errors occurring at the metaphase 1 stage (MI) which leads to aneuploidies in the embryo. Furthermore, the optimal luteal phase support has yet to be agreed upon (21) and ideas concerning early pregnancy management as well as the avoidance of pre-term delivery are only now appearing in the literature (31). The idea of evaluating a GH adjuvant trial must take these aspects into consideration to identify potential confounders.

PIVET EXPERIENCE WITH GH AS AN ADJUVANT IN IVF

Encouraged by the study on women aged >40 years which showed significant improvement of live birth rates by ovarian co-stimulation with GH in IVF (32), a GH adjuvant study was conducted at PIVET and the 5-year project was reported in 2010 (4). It was not an RCT but was designed as a prospective sequential crossover whereby patients identified as poor-prognosis were offered the option of using, or not using, GH in the forthcoming IVF cycle. (Some elected to use the expensive hormone in the immediate IVF cycle, others deferred depending on the outcome of a further non-GH cycle). Two protocols were explored using 10 IU ampoules given by injection in one of 2 protocols; Days 21 of previous cycle followed by Days 2, 6, 8, 10, and 12 of the IVF treatment cycle (60 IU over ∼20 days); or Days 7, 14 and 21 of the previous cycle followed by a final injection on Day 2 of the IVF cycle (40 IU over ∼35 days). Of 2,174 autologous IVF cycles during the period, 488 (22%) were classified poor-prognosis from previous experience providing 232 cycles started (initiated) with GH adjuvant and 256 without. The productivity rate was significantly higher among those poor-prognosis women given GH (43 vs. 11 live births; $p < 0.001$). However, the women classified as good prognosis had a productivity rate of 45.4% being well ahead of the poor-prognosis categories including those given GH 18.5%; $p < 0.0001$. From this study we understood that GH was safe for both mothers and offspring including the higher dosage regimen which encouraged

TABLE 3 | Listing adjustments, adjuvants, and add-ons which have been variously used in IVF and reported in medical literature.

Adjustments, adjuvants, and add-ons in IVF
i. Response to ovarian stimulation
a. Adjusting the schedule for LDR, Flare, and Antagonist protocols
b. Adjusting the FSH dosing regimen
c. Adding LH or HMG into the regimen
d. The consideration of Clomid, Tamoxifen, or Letrozole
ii. Ovulation trigger
a. HCG dosage
b. GnRha trigger
c. Double trigger
iii. Gamete preparation
a. Sperm preparation and pentoxifylline enhancement
b. Oocyte culture from GV to M-II (IVM)
c. Growth hormone for improved oocyte quality
d. Calcium ionophore A23187
e. ICSI despite normal semen analysis
iv. Embryo assistance
a. Zona hatching (mechanical, acid Tyrode, pronase solution, or laser method)
b. Embryo glue (hyaluronin with recombinant human albumin)
v. Luteal support protocols
a. HCG
b. Progesterone (P4) and progestogens
c. E2/P4 combination pessaries
d. GnRha
vi. Implantation enhancement
a. Low-dose aspirin ± heparin (anti-thrombotic)
b. Antibiotics
c. Enoxaparin/heparin
d. Oral steroids (ACA, ANA, and LA antibody suppression)
e. Verapamil (uterine relaxant, calcium antagonist)
f. Acupuncture (stress relief, possible uterine relaxant)
g. Atosiban (oxytocin/vasopressin Via receptor antagonist)
h. G-CSF and Filgrastim
i. C0-enzyme Q10
j. Dopamine agonists
k. Intralipid (enhancing mitochondrial function)
l. Testosterone (for low-androgen female)
m. DHEA (for low-androgen female)
n. Melatonin (strong anti-oxidant hormone)
o. Growth Hormone (multifarious and ubiquitous actions)
p. Endometrial scratch procedure
q. Platelet infusion to uterine cavity
r. Endometrial stimulation (GCSF-granulocyte colony stimulating factor)
s. Paternal Lymphocyte immunization
t. IVIG (intravenous Immunoglobulin)
vii. Embryo selection
a. Culture to blastocyst
b. Quality grading of blastocyst
c. Chromosomal evaluation for aneuploidy screening
viii. Embryo-Endometrial synchrony
a. Clinical calculation (Days from LH surge or P4 rise/ P4 supplements)
b. Endometrial assessment (Endometrial receptivity array; ERA)
ix. Early pregnancy supports
a. Progesterone and progestogens
b. Uterine relaxants (β_2-agonist e.g., ritodrine, salbutamol)
c. HCG (enhance corpus luteal function)
d. Low-dose aspirin (anti-thrombotic)
x. Technical options
a. Single lumen vs. flushing needle for follicle aspiration
b. Ultrasound control for embryo transfers
c. Myomectomy and pelvic surgeries pre-embryo transfers
d. Tubal vs. uterine embryo transfers

These adjuvants are categorized according to the putative area of action (noting HCG and Growth Hormone may apply in two areas—GH improving oocyte quality and possibly enhancing embryo implantation). Cochrane reviews have assessed all of these adjuvants and interventions, finding only aspirin and steroids demonstrating promising, potentially beneficial outcomes; but none yet proven to the highest level of EBM standards (26). Apart from one study including an "aspirin arm" and a second with a "DHEA" arm, none of the reported GH studies have considered these numerous potential confounders. G-CSF, granulocyte colony stimulating factor (filgrastim; G-CSF analog); GV, germinal vesicle; M-II, metaphase II; IVM, in vitro maturation; ACA, anti-cardiolipid antibodies; ANA, anti-nuclear antibodies; LA, lupus antibodies; LDR; long down regulation; FSH, LH, GnRHa in text.

its continued use. The benefits appeared similar from the two GH regimens described.

A second study from our PIVET facility was reported in 2017 (6) and again demonstrated an improvement in the quality of oocytes retrieved from 1,488 women categorized as poor-prognosis in IVF. The women were given dosages of 1.0 or 1.5 IU daily in the 6-week lead-up to OPU. This retrospective observational study showed a significantly higher oocyte utilization rate and embryo utilization rate among those women receiving GH compared to a computer-matched group of poor-prognosis cases who did not receive GH. This means a significantly higher number of oocytes become embryos which were either transferred as fresh ETs or cryopreserved for subsequent FET cycles. Among the case-matched women classified as poor-prognosis having fresh ETs the clinical pregnancy rate was 2.2-fold higher for Day-3 embryos and 7.6-fold higher for blastocyst transfers in those who had the GH adjuvant. This translated into an over-all improvement in live births of 6.2-fold for fresh ETs from the use of GH adjuvant (95% CI 2.8–13.4, $p < 0.001$). Of interest during this study a group of women classified as poor-prognosis chose the less-expensive oral Dehydroepiandrosterone (DHEA) option and others had combined DHEA with GH. In a separate report we failed to show any benefit from DHEA supplementation alone, neither any potentiating benefit or modification of the effect of GH treatment (33). Extending from this study, we subsequently reported on the outcome of treatments comparing the pregnancy outcomes of those cryopreserved embryos generated under GH influence with those arising without GH influence (34). Where FET cycles were carefully matched for age and poor-prognosis category, along with 6 other variables including embryo grading, AFC, AMH, BMI, and mid-luteal P4 levels, and analyzed by binary logistic regression, the live birth outcome was found to have improved significantly, being 2.7-fold higher (OR 2.71; $p = 0.02$) implying that GH had an influence beyond improving oocyte competence, extending to an embryo quality factor represented by enhanced competence to generate a live birth. Such embryos appeared morphologically similar to non-GH generated but

outcomes were significantly better. We have suggested that such GH-generated embryos have an improved placentation capacity. Future proposed studies should also explore whether those embryos have any reduction in aneuploidy rates. However, the idea of GH being able to "fix" the problem of aneuploidy completely lacks scientific evidence.

GLOBAL STUDIES WITH GH AS AN ADJUVANT IN ASSISTED REPRODUCTION

The PIVET experience has been placed in context with 42 reported GH adjuvant studies from the year 2000 and summarized in **Table 4** (4, 6, 32, 34–72). The first report in the table is from Nancy, France describing a single case with pan-hypopituitarism treated in a cross-over study (X-over refers to case studies where the outcomes were assessed on the same woman/women being treated with GH after cycles without GH). The woman was given GH 1 IU daily beginning 12 weeks prior to HMG stimulation and continuing through both the follicular stimulation phase and the luteal phase. GH was ceased following the detection of pregnancy 14 days after the HCG trigger injection (10,000 IU) with serum B-HCG of 462 IU/ml. A healthy male infant was delivered at 39 weeks with birthweight 3,780 g. This single case X-over report (35) should be compared with other cases of hypopituitarism such as the 4 cases from Rome, Italy (42) who had defined AGHD secondary to conditions such as brain trauma, empty Sella Turcica and Rathke's cyst. Normal fertility was established in these previously infertile women following "standard endocrine treatment" being weekly injections with GH averaging 0.5–1.0 IU daily given over a 3–6 month period, ceasing when pregnancy was diagnosed. In each of the 4 cases pregnancy outcomes were perfectly normal from all respects and the women successfully breast-fed their children. Two further single case X-over studies have also been reported, one from Brussels, Belgium (56) where a woman who had hypophysectomy and many IVF failures, conceived immediately from IVF following GH replacement therapy (∼1 IU daily over 6 weeks). She delivered healthy twins at the 38th week of gestation. Of interest it was noted that her endometrial thickness had improved markedly under GH replacement therapy. The other similar single-case was recently reported from Bucharest, Romania (71). A woman with AGHD who had repeated failures from IVF, was given GH daily for 3 months in the lead-up to a further IVF treatment, this time resulting in better quality oocytes and a live birth.

Seven of the reports in **Table 4** document the results of Systematic Reviews (Syst Rev) and Meta-analyses. The Cochrane report of 2003 (37) documented the outcomes from 9 studies undertaken prior to the year 2000 (i.e., studies pre-**Table 4**). Six of those studies were undertaken on women classified with POR and three had unspecified classification for poor- prognosis. The first analysis showed no significant improvements but when trials using GH as the only adjuvant, particularly excluding a study combining growth hormone releasing factor (GRF), were separately analyzed, there was a significant increase in livebirths from 3 RCTs (OR 4.37). Five of the further Systematic Reviews with Meta-analyses (39, 44, 45, 51, 61) all revealed improved oocyte and embryo utilization and 3 reported significantly higher live births (ORs 3.2, 5.4, and RR 1.9). However, two separate M-analyses (51, 61) showed no increase in live births although oocyte and embryo utilization were significantly improved (OR 0.8 and 1.5; and OR 1.9 and 1.5, respectively). The report from Anhui, China (51) comprised 20 GH studies including one pre-2000 and 12 studies from various locations in China, some reports not fully detailed in English. Others are confused by alternative spelling of lead author e.g., Guan vs. Qun (40) and selection of family name for lead author e.g., Xue-Li Li vs. Li X-L (57).

Excluding the 6 X-over reports and the single-case IVM study (38), there were 28 controlled studies, 19 of which were categorized as retrospective or prospective / observational and 12 were RCTs, with 10 describing strictly random allocations. Of the non-RCTs, two-thirds reported significantly elevated oocyte and embryo utilization rates which translated to increased pregnancy rates in most, but live-birth rates were significantly improved in only 50% of the studies (9 but 2 others reported increased pregnancy rates but did not record births). Three of the 9 non-RCT studies showing improved live births emanated from PIVET (4, 6, 34) and the potential reasons for the varied outcomes among the global studies will be discussed (below). From the 12 RCTs, oocyte and embryo utilization were significantly elevated in all but one study, sometimes with an increase in pregnancy rates but only 5 of the 12 studies reported any significant elevation in live birth rates (32, 45, 54, 60, 69), three of which were focussed on endometrial enhancement (54, 60, 69).

The one RCT which failed to show any benefits from the GH adjunct (70) deserves specific scrutiny, as it was a registered study with which PIVET participated. In 2010, a multicenter, double-blind placebo-controlled trial was established in Australia and New Zealand with 10 participating IVF centers. Women had to be younger than 41 years, with demonstrated POR from previous IVF and have body mass index not >32 kg/m^2 and baseline FSH level no higher than 15 IU/L. The trial was registered as the LIGHT (Live birth, *in-vitro* fertilization & GH Treatment) study and intended to recruit 390 IVF couples to provide 195 participants in each arm. This number would have provided statistical power at the 5% significance level if the live birth rate improved from a base level of 10% to an enhanced level of 20%. Both the GH hormone pen and the placebo pen were identical in appearance and both the patient as well as her medical attendants were blinded to the active vs. inactive injection. The GH dosage was 12 IU to be given concomitant with the gonadotropin, meaning approximately 12 days (actual range 11–13 days) of injections; ∼144 IU total GH. However, the LIGHT study closed after 8 years effort having recruited only 130 couples into actual treatment, being only a third of the number required. As we have earlier indicated, where patients are paying for treatment, they are reluctant to risk being allocated to the placebo arm, even though the GH hormone was provided without charge. They surmised that there would be a loss of monies and opportunity, particularly if they were aged in their late thirties. Nonetheless, the data from the 130 women has been analyzed and published (69)

TABLE 4 | Forty-two studies published in English from year 2000 utilizing growth hormone in IVF programs.

References *Spec feature*	Type of study	GH v Con'l	GH dose	GH dur'n	Oocyte utilis'n	Embryo utilis'n	Pregnancy	Live births
Salle et al. (35) *Hypopituitarism*	X-over	1 v 1	1 IU d	3 m	↑	↑	↑	↑
Sugaya et al. (36) *PORs*	X-over	9 v 9	4 IU d	4 w	↑	↑	ns	ns
Harper et al. (37) *Cochrane*	Syst Rev + M-analysis	154 v 150	4–8 IU daily	1–2 w	↑	↑	↑ OR 1.8	↑ OR 4.4*
Tesarik et al. (32) *women >40 y*	RCT	50 v 50	8 IU d	1 w	ns	ns	↑ $P < 0.05$	↑ $P < 0.05$
Menezo et al. (38) *GH in vitro*	Single case GV oocytes	GH to IVM	1.6 IU	stat	13/14 to MII	9/13 2PNs	1 B/C to FET	Healthy baby
Kyrou et al. (39)	Syst Rev + M-analysis	42 v 40	12–28 IU alt d	2–3 w	↑	↑	↑ OR nr	↑ OR 5.2
Guan et al. (40) *GH + aspirin*	RCT	20 v 20	nr	nr	↑	↑	ns	ns
Kucuk et al. (41)	RCT	31 v 30	12 d	3 w	↑	↑	ns	ns
Giampietro et al. (42) *GHD cases*	Observ	4 cases	3–6 IU w	6–12 m	Spont	Spont	Spont X4	Spont X4
Hazout et al. (43) *PORs*	Observ	245 v 2780	8 IU d	2 w	↑	↑	OR 1.5 $P < 0.01$	nr
Kolibianakis et al. (44)	Syst Rev + M-analysis	83 v 80	4–24 IU alt d	1–3 w	↑	↑	↑ OR 2.8	↑ OR 3.2
Yovich and Stanger (4) *Poor prognosis*	Prospec X-over	221 v 241	3–3.5 IU d	3 w or 6 w	ns	ns	↑ $P < 0.001$	↑ $P < 0.001$
Duffy et al. (45) Cochrane	Syst Rev + M-analysis	148 v 131	4-8 IU d 24 alt d	1-3 w	↑	↑	↑ OR 3.3	↑ OR 5.4
Eftekhar et al. (46)	RCT	40 v 42	4 IU d	2 w	↑	↑	ns	ns
Haydardedeoglu et al. (47)	Retrospec	37 v 44	1.5–3 IU d	3 w	ns	ns	↑ $p < 0.001$	↑ $p < 0.002$
Hu et al. (48) PORs	Retrospec	102 v 287	4 IU	2 w	↑	↑	ns	ns
Lattes et al. (49) *PORs*	X-over	64 v 64	0.5 IU d	2 w	↑	↑	ns	ns
Dunne et al. (50) *Luteal phase GH*	Retrospec	14 v 28	10 IU d	2 w Pre IVF	ns	ns	ns	ns
Yu et al. (51)	Syst Rev + M-analysis	613 v 3,175	2–9 IU d average	1–3 w	↑	↑	ns	ns
Bayoumi et al. (52) *Microflare stim'n*	RCT	72 v 73	8 IU d	2 w	↑	↑	nr	nr
Bassiouny et al. (53)	RCT	68 v 73	8 IU d	1 w	↑	↑	ns	ns
Wang et al. (54) *FET cycles HRT*	RCT (not strict)	77 v 77 v 76	4 IU d	2 w	↑ endometrial thickness		↑ $P < 0.03$	↑ $P < 0.03$
Du et al. (55) *NORs*	Retrospec	556 v 558	4.5 d	1 w	↑	↑	↑ OR 3.2	nr
Drakopolous et al. (56) *Hypopituitarism*	X-over	1 × 1	1 IU d	6 w	oocytes ↑ end thickness		↑ Twin preg	↑ 38 w
Ob'edkova et al. (57) *PORs*	Prospec observ	25 v 25	4 IU d	2 w	↑	↑	↑ OR 9.1	nr
Ho et al. (58) *3 arms: Age ≥40 y, RIF, PORs*	Retrospec Matched controls	98/36 118/118 33/33	3 IU d 2 IU d 2 IU d	2 w 2 w 2 w	Ns ↑ ↑	Ns ↑ ↑	ns $p < 0.01$ ↑ $p < 0.01$	nr nr nr
Keane et al. (6) *poor prognosis*	Retrospec	161 v 239	1–1.5 IU/d	6 w	↑	↑	↑ RR 3.4	↑ RR 6.2
Li et al. (59)	Syst Rev + M-analysis	320 v 343	1–12 IU d	1–3 w	↑	↑	↑ RR 1.8	↑ RR 1.9

(Continued)

TABLE 4 | Continued

References *Spec feature*	Type of study	GH v Con'l	GH dose	GH dur'n	Oocyte utilis'n	Embryo utilis'n	Pregnancy	Live births
Altmae et al. (60) *RIF donor oocyte*	RCT	35, 35 v 35	nr	2 w	↑ endometrial thickness		↑ OR 6.9	↑ OR 6.4
Hart et al. (61)	M-analysis	351 v 352	M-analysis	1-2 w	↑ OR 1.9	↑ OR 1.5	↑ OR 1.5	ns
Cai et al. (62) *PORs*	Retrospec X-over	41/380 v 41/380	2 IU	6 w	↑	↑	ns	↑ $p < 0.003$
Dakhly et al. (63) *PORs*	RCT	120 v 120	8 IU d	3 w	↑	↑	ns	ns
Chen et al. (64) *RIF cases*	Observ	22 v 20	2 IU d	2 w	↑	↑	↑ $p < 0.05$	↑ $p < 0.05$
Chu et al. (65) *Mild stiml'n*	Retrospec	61 v 71	2 IU d	2 w	↑	↑	ns	ns
Choe et al. (66) *Sust. Release GH*	RCT	64 v 63	S-R GH ~3 IU d	4 w	↑	↑	ns	ns
Regan et al. (67) *GC study*	Retrospec	13 v 10	2.5–3 IU d	3 w	↑	↑	↑	ns
Safdarian et al. (68) *PORs*	Retrospec	34, 32 v 26	0.5 IU –2.5 d	5d & 20 d	↑	↑	↑	ns
Cui et al. (69) *Thin endometrium*	RCT (?) FET cycles	40 v 53	?	2 w	↑ endometrial thickness		↑	↑
Norman et al. (70) *- recruited 130 of intended 390*	Double blind RCT	65 v 65	12 IU d	2 w	ns	ns	ns	ns
Keane et al. (34) *FETs*	Retrospec	109 v 201	1–3 IU d	3–6 w	↑	↑	↑ OR 1.8	↑ OR 2.7
Albu et al. (71) *AGHD case*	X-over	1 v 1	1 IU d	3 m	↑	↑	↑	↑
Liu et al. (72) *NORs*	Retrospec Matched	781 v 781	2 IU d 4 IU d	2 w 6 w	ns	ns	↑	nr

**Harper et al. (37); 6 studies, OR highest when Howles 1999 GHRF study removed (OR 2.4–4.4). RCT, randomized controlled study; ns, not significant; nr, not reported; spont, spontaneous; Prospec, prospective; retrospec, retrospective; utilis'n, utilization; cont, control; trig, trigger; Observ, observational study; d, day; w, week; m, month; y, year; GC, granulosa cell; FET, frozen embryo transfer; RIF, recurrent implantation failure; NOR, normal ovarian responder.*

with the report showing no improvement in oocyte number or utilization; no improvement in embryo number or utilization and no difference in either pregnancy rate or live birth rate. The only difference demonstrated between the two arms of the trial was the finding that GH patients reached oocyte retrieval faster than non-GH patients, similar to that shown in other earlier studies (44, 60). This may indicate that GH has an effect on folliculogenesis; however, this possibility was not supported by differences in embryo quality. Nonetheless, the links between GH and folliculogenesis, oocyte quality and responsiveness to gonadotrophins is still unclear from this study, being underpowered, focussed only on POR cases. That study does not yet report outcomes from cryopreserved embryos, although we would not hold high hopes for these as they were all cryopreserved in slow-freeze protocols prior to the introduction of vitrification to Australian IVF facilities. The authors acknowledge these points but also conclude with a negative comment*: "In conclusion, this study does not show increased efficiency of human GH as an adjunct to FSH treatment in subjects receiving IVF who have been previous poor responders." They caution women against expenditure in this area, citing their own earlier Meta-analysis which showed no pregnancy or live birth benefit for POR cases* (61). Furthermore, the National Institute for Health and Care Excellence (Nice) has issued clinical guideline CG156 recommending "Do not use growth hormone or dehydroepiandrostenedione (DHEA) as adjuvant treatment in IVF protocols" (73). This recommendation is based on a dogmatic EBM attitude that an appropriately structured RCT has not demonstrated a benefit; however we would argue that such a study is not feasible in the current circumstances.

Our perspective is described in response to another recent study, that from the well-published group from Cairo, Egypt (63). Their registered RCT, albeit with borderline numbers (120 in each arm), trialed a 3-week course of GH 7.5 IU daily in POR cases. They showed a significant improvement in both oocyte utilization and embryo utilization, meaning more embryos were transferred or cryopreserved in the GH arm. However, these improvements did not translate into more pregnancies or more livebirths, either from fresh cycles or from the added FETs (cumulative live births; live birth productivity rate). In a letter to the same journal we pointed out several limitations which could have limited their outcomes (74).

OPTIMIZING IVF OUTCOMES; A SINGLE-CENTER VIEWPOINT

We would summarize the current requirements to maximize the opportunity for embryos to implant and achieve higher LBRs, thereby reducing the poor-prognosis rate in IVF, requires adherence to the previously mentioned protocols (21), namely:

1. Apply PIVET FSH dosing Algorithms to optimize oocyte numbers (at 10 ± 2)
2. Apply SET protocol for all cycles, especially the fresh cycle.
3. Blastocyst culture preferred, with best quality embryos vitrified by Cryotop method
4. Strong luteal support in fresh cycle using P4 pessaries $\pm$ HCG with monitoring, enabling adjustment of dosages. Optimal early-luteal serum progesterone levels should range from 60 to 100 nmol/l (75) rising to between 150 and 250 nmol/l in the mid-luteal phase. Sometimes P4 injections will be required
5. FET cycles conducted under either natural cycles or HRT (for logistic benefits). Optimal P4 levels should be between 60 and 100 nmol/l in the mid-luteal phase (76)
6. FET with SET preferred with PIVET regimen: P4 Pessaries $\pm$ P4 injections
7. GH adjuvant therapy for IVF cases diagnosed with AGHD.

The last point (#7) may be considered controversial with strong skepticism about GH use expressed by several prominent clinicians in the field (70, 77, 78) excepting when used for women with hypopituitarism. Such can be due to a range of anatomical causes such as empty Sella Turcica, pituitary adenomas, Rathke's cyst/pouch, hypophysectomy, and other intracranial trauma as well as medical conditions such as Sheehan's syndrome. Such cases respond dramatically well to growth hormone replacement therapy 1 IU daily for 3–6 months as shown from the 6 X-over studies in **Table 4**. On the other hand, equally prominent IVF clinicians (79) are perplexed that GH is under-utilized given that "the most recent Meta-analysis (59) shows almost double live birth rates in those poor responders and/or couples with a reduced prognosis."

CONCLUDING VIEWPOINT

Our concluding viewpoint is that GH is clearly indicated in those women with infertility where this can be shown to be due to AGHD. This condition is currently under-diagnosed, but can be determined by applying screening tests involving IGF-1 and its main binding protein IGFBP3 (8). Endocrinologists may utilize sophisticated challenge tests where the diagnosis is uncertain, but IVF specialists may apply a simpler screening where there is already clinical evidence such as advanced female age and repetitive failure to generate any blastocysts for vitrification (i.e., defined poor-prognosis). The Bologna screening of POR has several limitations with respect to the application of GH, many of which have been discussed earlier in this article. In particular POR may often represent a highly depleted ovarian reserve, and such is an impossible challenge for GH and the inclusion of such cases may well explain the variable outcomes of the GH trials indicated in **Table 4**. We would believe that GH would apply best to those women who do respond to high-dose gonadotrophin injections (generating 8–12 oocytes with FSH doses up to 450 IU), but who fail to generate sufficient blastocysts of suitable quality to enable at least one or two for cryopreservation, after the transfer of one embryo in the fresh cycle. Where a freeze-all option is contemplated, there should be at least 2 high-grade embryos cryopreserved, otherwise the case can be classified as poor-prognosis, warranting GH therapy. With respect to the dosage and duration of GH therapy, our experience over more than 12 years indicates that 1 IU daily is sufficient to produce a response (e.g., raising IGF-1 levels) but an optimum response with respect to oocyte quality probably requires 4–6 months to cover the full period of folliculogenesis from the earliest stage of primary follicle recruitment being at least 20 weeks prior to the ovulatory cycle, when paracrine controls over oocyte development are strongest (80). We acknowledge there may be both logistic and financial problems to such a prolonged treatment schedule, hence a compromise treatment proposal could be a six-week schedule, beginning Day 2 of the menstrual cycle preceding the IVF cycle. Perhaps an increased dosage of GH at 2 IU might be a rational consideration given that the pioneer studies in the 1980's showed a dose-related effect on both follicle growth and IGF-1 levels (8, 81). A further notion that GH may help to preserve the primary follicle pool is appealing but awaits specific research. Such an idea implies that older women (e.g., >35 years) who wish to preserve their fertility, might benefit from continuous long-term GH therapy. Whilst studies investigating this idea have never been reported, there are reports that women who had GHD as children, will have health and fertility benefits from continuing GH therapy after puberty, the current stage of cessation (8). We believe there is sufficient data currently showing that GH can have a beneficial effect in IVF programmes but further research is required to forecast which woman will be deemed poor-prognosis, how such may be prevented, which cases will benefit from GH, and what therapeutic regimen should be applied for optimal management.

AUTHOR CONTRIBUTIONS

JY heads this research team involving Clinical (JY and YY) and laboratory studies (SR and KK). Data analysis and manuscript preparation is shared by JY and KK.

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Low Dose Growth Hormone Adjuvant Treatment With Ultra-Long Ovarian Stimulation Protocol in Poor Responders Showed Non-inferior Pregnancy Outcome Compared With Normal Responders

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Background: Growth hormone (GH) has long been used as adjuvant treatment in ovarian stimulation for *in vitro* fertilization (IVF), especially in poor responder (PR) patients. However, its clinical efficacy remains unclear, and most studies are underpowered owing to their small sample size with different regimens.

Methods: Our study was divided into two parts. The first part was a parallel randomized, observational study in which 184 patients who fulfilled the criteria of poor ovarian response (POR) were enrolled and received ultra-long ovarian stimulation protocol with or without GH adjuvant therapy. For the second part, clinical data were retrospectively extracted from 163 patients classified as PRs who received 10 IU GH adjuvant therapy and 157 patients classified as normal responders (NRs) who received the same IVF protocol treatment without GH adjuvant therapy.

Results: For the first part of the study, the ovarian response, the number of oocytes retrieved, and the number of available embryos transferred were all significantly higher in the GH (+) group than in the GH (–) group. The clinical pregnancy rate was significantly higher in the GH (+) group (31.9 vs. 16.7%, $p = 0.0168$). The miscarriage rate did not differ significantly between the groups. The ongoing pregnancy rate was also significantly higher in the GH (+) group than in the GH (–) group (26.6 vs. 14.4%, $p = 0.0418$). Logistic regression revealed that the chance of clinical pregnancy in the GH (+) group significant increased 2.34-fold in comparison with the GH (–) group ($p = 0.018$). Subgroup analysis showed that the chance of clinical pregnancy in the GH (+) group significantly increased 2.38-fold ($p = 0.034$). The second part of the study showed no statistical difference between the PR with GH and the NR without GH groups regarding the implantation rate (15.6 vs. 19.8%, $p = 0.3254$) and the clinical pregnancy rate (31.9 vs. 39.5%, $p = 0.1565$). The NR without GH group showed insignificantly higher chance of clinical pregnancy (OR = 1.39, $p = 0.157$) compared with the PR with GH group.

Conclusion: Our results suggested that low-dose GH supplementation may improve ovarian response and pregnancy outcome in POR patients, particularly in patients younger than 40 years old. Moreover, the low-dose GH effect in POR patients resulted in non-inferior clinical pregnancy outcome compared with NRs.

Keywords: growth hormone, low dose, poor ovarian response, *in vitro* fertilization, adjuvant treatment, pregnancy outcomes

INTRODUCTION

Controlled ovarian hyperstimulation (COH) has long been a crucial part of *in vitro* fertilization (IVF), along with the development of assisted reproductive technologies (ART). The goal of COH is to recruit multiple follicles and to obtain many mature oocytes to increase chances for conception (1). Poor ovarian response (POR) to ovarian stimulation indicates a reduction in follicular response and a reduced number of retrieved oocytes. Despite using different stimulation protocols and multiple treatment courses of IVF, the pregnancy outcome remains poor in POR patients, which is frustrating for both the patients and the clinicians.

The incidencevs of POR to ovarian stimulation reportedly ranges from 9 to 24% of IVF-embryo transfer (ET) cycles, according to various studies (2, 3). POR, or poor responders (PRs), remains a significant challenge for IVF practice owing to not only the heterogeneity of the pathophysiology but also the lack of general consensus in the definition of POR. The latter directly leads to poor literature quality with insufficient evidence to identify any particular intervention to improve outcomes in POR patients. The definition of POR was under debate without uniform agreement for decades until the European Society of Human Reproduction and Embryology conducted a consensus study and reached a definition for POR in 2011. This so-called Bologna criterion defines poor response in IVF as comprising at least two of the following three features: (i) advanced maternal age (≥40 years) or any other risk factor for POR, (ii) previous POR (≤3 oocytes with a conventional stimulation protocol), and (iii) an abnormal ovarian reserve test [antral follicular count (AFC) < 5–7 follicles or anti-mullerian hormone (AMH) < 0.5–1.1 ng/ml]. Two episodes of POR after maximal stimulation are sufficient to define a patient as a PR in the absence of advanced maternal age (4).

Numerous studies have been conducted using different interventions for the management of POR. Among them, the use of growth hormone (GH) as an adjuvant treatment with gonadotropins to facilitate follicular development and ovulation induction was first introduced by Homburg et al. in 1988 (5). GH is an anabolic peptide hormone which functions to increase cell growth and proliferation, and it has been reported to modulate the action of follicle-stimulating hormone (FSH) by binding to GH receptors on granulosa cells to increase the synthesis of insulin-like growth factor-I (IGF-I). IGF-I augments the effect of gonadotropin action on both the granulosa and theca cells and plays an essential role in follicular development, oocyte maturation, and steroidogenesis. It can also improve follicular survival and granulosa cell proliferation by directly inhibiting follicle apoptosis (5–9). Nevertheless, results from previous studies are controversial regarding the effect of GH as an adjuvant therapy during COH. GH has been demonstrated to increase the retrieved oocytes and improve embryo quality and pregnancy outcomes in several studies (10–19) and meta-analyses (2, 3, 20–22). However, several clinical trials failed to demonstrate significant benefits regarding clinical pregnancy and live birth rates (23–27). The small sample size of the trials, inconsistency in the definition of POR, and different stimulation protocols with GH regimens all contributed to the bias of the outcomes. Therefore, the true value of GH adjuvant treatment remains elusive to date.

Very few reports are available that discuss the influence of GH dosage and regimen. Furthermore, no previous study using an ultra-long down-regulation protocol combined with GH adjuvant treatment has been reported. In our study, we aimed to investigate the efficacy of low-dose GH adjuvant treatment in PR patients compared with normal responders (NRs) using an ultra-long ovarian stimulation protocol.

MATERIALS AND METHODS

Study Period and Participants

This parallel randomized, observational cohort study was conducted in a single IVF center in Taipei Medical University Hospital from January 2010 to October 2012.

The study was divided into two parts. First, enrolled patients were classified as PRs who had fulfiled at least two of the following criteria: (i) advanced maternal age (≥ 40 years old) or any other risk factors for POR, (ii) previous episode of POR (≤3 mature oocytes retrieved with a conventional stimulation protocol), and (iii) an abnormal ovarian reserve test (AFC < 5–7 follicles or AMH < 0.5–1.1 ng/ml) between January 2010 and November 2011. Second, the data of patients who were classified as PRs who received GH adjuvant therapy and as NRs who received the same IVF treatment protocol without GH adjuvant therapy were collected between January 2012 and October 2012.

Clinical Management

To prevent possible bias from different physicians, all patients were handled by a single clinician. In addition, to eliminate bias from fresh vs. frozen embryo transfer, only the first IVF cycle with fresh embryo transfer (ET) within the study period was

analyzed. All patients who participated in the study followed a gonadotropin-releasing hormone (GnRH) agonist ultra-long IVF protocol. In brief, the patients received a half-dose one-shot long-acting GnRH agonist (leuprolide acetate, 1.88 mg) at cycle day 1–3, followed by ovulation induction with gonadotropin starting between day 35 and 40. The dosage of gonadotropin was adjusted according to the ovarian response, which was monitored by transvaginal ultrasonography and serum hormone level. When two or more follicles reached a diameter of 17–18 mm, 6,500–10,000 IU of human chorionic gonadotropin (hCG) was administered, and transvaginal oocyte retrieval was performed 34–36 h later. Fertilization was conducted by intracytoplasmic sperm injection (ICSI) ∼2 h later, after oocyte denudation. Fresh day 3 ET was performed with the best-quality blastomeres.

The first part of the study was a parallel, randomized study. Patients who fulfilled the abovementioned inclusion criteria were randomly allocated into two groups (named the GH (+) group and the GH (–) group) with simple randomization using a tossing coin method. Patients in the GH (+) group ($n = 94$) received co-treatment with GH adjuvant therapy (Saizen; Merck Serono) at a dosage of 4, 4, and 2 IU for three successive days, along with the ovulation induction. The total GH dosage was 10 IU for each patient in the GH (+) group. Patients in the GH (–) group ($n = 90$) received the same IVF protocol without GH adjuvant therapy.

For the second part of the study, patients who were classified as PRs and received co-treatment with GH adjuvant therapy ($n = 163$) and NR patients ($n = 157$) who received the same IVF protocol without GH adjuvant therapy were enrolled.

Data Analysis and Statistics

Clinical parameters including age, AMH level, E2 level on the day of hCG administration, total gonadotropin dosage, mean number of oocytes retrieved, number of embryos transferred, embryo quality, and number of surplus embryos were recorded and analyzed. The main outcomes of the study were implantation rate, clinical pregnancy rate, miscarriage rate, and ongoing pregnancy rate. The implantation rate was calculated as the ratio of the number of gestational sacs to the number of embryos transferred. Clinical pregnancy was defined as the presence of a positive heartbeat in a healthy gestational sac, detected by transvaginal ultrasound 4–5 weeks after embryo transfer. Early miscarriage was defined as pregnancy loss before 12 weeks of gestation. Ongoing pregnancy was defined as viable pregnancy after 20 weeks of gestation.

Data are presented as mean and SD for quantitative variables and percentage for qualitative variables. The odds ratio (OR) and 95% confidence interval were calculated for clinical pregnancy rate and miscarriage rate. Comparisons between groups were performed using the Student's *t*-test for quantitative variables and the chi-square test for qualitative variables. SPSS (IBM Statistics, ver. 25) was used for all statistical analyses. A $p < 0.05$ was considered statistically significant.

TABLE 1 | Clinical parameters and outcomes of poor-responders with GH (+) and GH (–) groups.

	GH (+)	GH (–)	*P*-value
N	94	90	
AMH, ng/ml	1.1 ± 0.4	1.1 ± 0.9	1.0000
Age, year	38 ± 3.4	37.1 ± 3.8	0.0919
E2 level on hCG day, pg/ml	679 ± 459	457 ± 357	0.0003*
No. of oocytes retrieved	5.5 ± 3.3	2.1 ± 0.7	<0.0001*
No. of embryos transfer	2.6 ± 0.9	1.7 ± 0.7	<0.0001*
Clinical pregnancy, *n* (%)	30/94 (31.9%)	15/90 (16.7%)	0.0168*
Miscarriage, *n* (%)	5/30 (16.6%)	2/15 (13.3%)	0.7755
Ongoing pregnancy, *n* (%)	25/94 (26.6%)	13/90 (14.4%)	0.0418*

*All values presented as mean ± SD, unless stated otherwise. *p < 0.05, statistically significant.*

RESULTS

Outcomes of PR Patient With GH (+) and GH (–) Groups

The clinical parameters and main outcomes in both groups are listed in **Table 1**. The baseline characteristics of both groups including age and AMH did not differ statistically. The E2 level on the hCG day was significantly higher in the GH (+) group (679 ± 459 vs. 457 ± 357, $p = 0.0003$). The number of oocytes retrieved was also significantly higher in the GH (+) group than in the GH (–) group (5.5 ± 3.3 vs. 2.1 ± 0.7, $p < 0.0001$), both indicating a higher ovarian response to stimulation in the GH (+) group. The number of embryo transfer was significant higher in the GH (+) group than in the GH (–) group (2.6 ± 0.9 vs. 1.7 ± 0.7, $p < 0.0001$). The clinical pregnancy rate was significantly higher in the GH (+) group than in the GH (–) group (31.9 vs. 16.7%, $p = 0.0168$; OR = 2.34, $p = 0.0177$), and the number of ET cycles was significantly higher in the GH (+) group (2.6 ± 0.9 vs. 1.7 ± 0.7, $p < 0.0001$). The miscarriage rate did not differ significantly between the groups. The ongoing pregnancy rate was also significantly higher in the GH (+) group than in the GH (–) group (26.6 vs. 14.4%, $p = 0.0418$).

We further subdivided both patient cohorts by age below and above 40 years old. The results showed that the E2 level on the hCG day was significantly higher in the GH (+) group (619 ± 317 vs. 449 ± 366, $p = 0.0065$) in patients under 40 years old but not in patients older than 40 years (685 ± 408 vs. 503 ± 336, $p = 0.0716$). Logistic regression revealed that the chance of clinical pregnancy in the GH (+) group significantly increased 2.34-fold ($p = 0.018$) in univariable analysis and 2.52-fold ($p = 0.011$) in multivariable analysis, respectively (**Table 2**). Subgroup analysis showed that the chance of clinical pregnancy in the GH (+) group significantly increased 2.38-fold ($p = 0.034$) in patients <40 years old, but not in patients more than 40 years old (OR = 3.23, $p = 0.17$; **Table 3**).

Outcomes of PR Patients With GH and NR Patients Without GH

We compared the clinical parameters and main outcomes of PRs with GH co-treatment and NRs without GH co-treatment

TABLE 2 | Logistic regression with univariate and multivariate analysis of the pregnancy outcome for poor-responders.

Variable		Clinical pregnancy rate, *N* (%)	Clinical pregnancy OR (95% CI)			
			Univariable analysis (Crude OR)	*P*-value	Multivariable analysis (Adjusted OR)	*P*-value
Growth Hormone	GH (+)	30 (31.9%)	2.34 (1.16–4.74)	0.018*	2.52 (1.23–5.16)	0.011*
	GH (–)	15 (16.7%)	1.00		1.00	
Age	<40	36 (28.8%)	2.25 (1.00–5.04)	0.05	2.46 (1.08–5.62)	0.032*
	≥40	9 (15.2%)	1.00		1.00	

*$p < 0.05$.

TABLE 3 | Subgroup analysis of the pregnancy outcome for poor-responders.

Variable	Subgroup	Clinical pregnancy rate, *N* (%)	Clinical pregnancy OR (95% CI)	*P*-value
GH (+)	Age < 40	23 (37.7%)	2.25 (0.84–6.03)	0.106
	Age ≥ 40	7 (21.2%)	1.00	
GH (–)	Age < 40	13 (20.3%)	3.06 (0.63–14.64)	0.162
	Age ≥ 40	2 (7.7%)	1.00	
Age < 40	GH (+)	23 (37.7%)	2.38 (1.07–5.28)	0.034*
	GH (–)	13 (20.3%)	1.00	
Age ≥ 40	GH (+)	7 (21.2%)	3.23 (0.61–17.10)	0.17
	GH (–)	2 (7.7%)	1.00	

*$p < 0.05$.

TABLE 4 | Clinical parameters and outcomes of poor-responder with GH co-treatment and normal-responder without GH co-treatment groups.

	PR with GH (+)	NR with GH (–)	*P*-value
N	163	157	
Age, years	38.8 ± 4.1	35 ± 3.8	<0.0001*
AMH, ng/ml	1.6 ± 1.4	3.6 ± 2.7	<0.0001*
Total FSH, IU	2495 ± 915	2160 ± 513	0.0001*
E2 level on hCG day, pg/ml	872 ± 723.6	1652 ± 1141	0.0001
No. of oocytes retrieved	5.8 ± 4.1	10.3 ± 6.0	<0.0001*
No. of good embryos	3.8 ± 2.7	7.0 ± 4.3	<0.0001*
No. of embryos transfer	2.5 ± 1.0	2.6 ± 1.8	0.5375
No. of surplus embryos frozen	0.7 ± 1.7	3.0 ± 3.8	<0.0001*
Implantation rate	15.6%	19.8%	0.3254
Clinical pregnancy rate, *n* (%)	52/163 (31.9%)	62/157 (39.5%)	0.1565

*$p < 0.05$.

(**Table 4**). The PR with GH [PR + GH (+)] patients were significantly older than the NR without GH [NR + GH (–)] patients (38.8 ± 4.1 vs. 35 ± 3.8, $p < 0.0001$), with significantly lower AMH (1.6 ± 1.4 vs. 3.6 ± 2.7, $p < 0.0001$). During ovulation induction, the PR + GH (+) patients required higher total gonadotropin (2495 ± 915 vs. 2160 ± 513, $p = 0.0001$), but had lower E2 level on hCG day (872 ± 723.6 vs. 1652 ± 1141, $p = 0.0001$) compared with the NR + GH (–) patients. The number of oocytes retrieved, the number of good-quality embryos, and the number of surplus frozen embryos were all significantly lower in the PR + GH (+) group than in the NR + GH (–) group. However, there was no statistical difference between the two groups regarding the number of embryos transferred (2.5 ± 1.0 vs. 2.6 ± 1.8, $p = 0.5375$), the implantation rate (15.6 vs. 19.8%, $p = 0.3254$), and the clinical pregnancy rate (31.9 vs. 39.5%, $p = 0.1565$).

Logistic regression revealed that the chance of clinical pregnancy in patients <40 years old significantly increased 2.41-fold ($p = 0.003$) in univariable analysis and 2.33-fold ($p = 0.006$) in multivariable analysis, respectively (**Table 5**). The NR + GH (–) group showed a slightly higher chance of clinical pregnancy (OR = 1.39, $p = 0.157$ in univariable; OR = 1.08, $p = 0.76$ in multivariable analysis, respectively), but these differences were insignificant (**Table 5**). Subgroup analysis showed that the chance of clinical pregnancy in the GH (+) group significantly increased 2.13-fold ($p = 0.033$) in patients <40 years old compared with patients more than 40 years old. The chance of clinical pregnancy showed an insignificant increase in patients <40 years old (OR = 1.14, $p = 0.63$) and an insignificant decrease in patients more than 40 years old (OR = 0.78, $p = 0.72$) (**Table 6**).

DISCUSSIONS

The first part of our study demonstrated that, in POR patients, low-dose GH supplementation significantly increased ovarian response, the number of oocyte retrieved, clinical pregnancy rate, and the ongoing pregnancy rate. The number of embryo transferred was also significant increase, which was mainly due to more available embryos obtained after GH supplementation. These results echoed the conclusions of previous studies and several meta-analyses which reported increased ovarian response and pregnancy outcome after GH supplementation. Using logistic regression and subgroup analysis, our results demonstrated that the effect of GH seemed more prominent in younger patients (<40 years old).

In the second part of our study, the POR patients were assumed to have poor prognosis because they were apparently older and had a significantly lower ovarian reserve than the NRs. POR patients with low-dose GH supplementation required a higher gonadotropin dosage but ended up with lower ovarian response, smaller oocyte yields, fewer good-quality embryos, and fewer surplus frozen embryos. However, the implantation rates and clinical pregnancy rates were comparable between

TABLE 5 | Logistic regression with univariate and multivariate analysis of pregnancy outcome for poor-responder (PR) with GH co-treatment and normal-responder (NR) without GH co-treatment groups.

Variable		Clinical pregnancy rate, *N* (%)	Clinical pregnancy OR (95% CI)			
			Univariable analysis (Crude OR)	*P*-value	Multivariable analysis (Adjusted OR)	*P*-value
Growth Hormone	PR (*N* = 163) GH (+)	52 (31.9%)	1.00		1.00	
	NR (*N* = 157) GH (–)	62 (39.5%)	1.39 (0.88–2.20)	0.157	1.08 (0.66–1.77)	0.76
Age	<40 (*N* = 234)	95 (40.6%)	2.41 (1.36–4.27)	0.003*	2.33 (1.27–4.29)	0.006*
	≥40 (*N* = 86)	19 (22.1%)	1.00		1.00	

*$p < 0.05$.

TABLE 6 | Subgroup analysis of the pregnancy outcome for poor-responder (PR) with GH co-treatment and normal-responder (NR) without GH co-treatment groups.

Variable	Subgroup	Clinical pregnancy rate, *N* (%)	Clinical pregnancy OR (95% CI)	*P*-value
PR, GH (+), *N* = 163	Age < 40	36 (38.7%)	2.13 (1.06–4.28)	0.033*
	Age ≥ 40	16 (22.9%)	1.00	
NR, GH (–), *N* = 157	Age < 40	59 (41.8%)	3.12 (0.85–11.43)	0.086
	Age ≥ 40	3 (18.8%)	1.00	
Age < 40, *N* = 234	PR, GH (+)	36 (38.7%)	1.00	0.63
	NR, GH (–)	59 (41.8%)	1.14 (0.67–1.95)	
Age ≥ 40, *N* = 86	PR, GH (+)	16 (22.9%)	1.00	0.72
	NR, GH (–)	3 (18.8%)	0.78 (0.20–3.08)	

*$p < 0.05$.

POR patients with low-dose GH adjuvant treatment and NRs without GH.

GH exerts biological effects on most of the tissue, including tissue metabolism and induced local synthesis of IGF-I to facilitate cell growth (8). In ovarian function, GH is necessary for oogenesis and folliculogenesis, which is fundamental for optimal female fertility (28). In the second part of study, we believed that the effect of GH compensated the poor-prognosis of this group and brought about comparable, or at least non-inferior, pregnancy outcomes compared with the NRs. Nevertheless, we could not rule out the beneficial effect of GH on other aspects, for example, the endometrium, that influenced pregnancy outcomes, since the NR group still possessed more oocytes and a higher embryo yield. The uterus is also a site of both GH production and GH action (28). The glandular cells of human endometrium and decidual tissue express GH receptors from the late luteal phase throughout pregnancy. Therefore, GH is speculated to play an important role in implantation (29). Previous studies also reported that GH might improve clinical outcome by increasing endometrial blood perfusion and improving endometrial receptivity during frozen-thawed ET in patients with repeated implantation failure (30, 31).

There is no standard protocol regarding GH regimen and dosage to date. The use of GH ranges from 4 to 24 IU, depending on different studies, and is usually started from previous cycle day 21 until the day of hCG administration. It is usually injected daily or on alternate days (2, 20, 21). Most studies have shown a positive impact of GH on ovarian response and pregnancy outcome, without adverse effect reported. Nevertheless, GH may have a detrimental effect on insulin resistance, and high GH levels can inhibit fertility and promote neoplasm; the exact threshold dosage is still unclear (28). Moreover, an increased economic burden is inevitable since GH is very expensive. To our best knowledge, ours is only the second low-dose GH supplementation study in the literature. Lattes et al. (14) conducted a self-controlled study of 64 PRs who failed to reach pregnancy in the previous cycle and were the first to use a GnRH agonist long protocol co-treatment with low-dose GH supplementation (0.5 IU/day) from previous cycle day 21 until the day of hCG injection. The average day of COH was 11.2 days, and the estimated total dose of GH was about 9.5–10 IU, almost the same as in our study. However, in addition to a different study design, the regimen we used required injections for only 3 days. Furthermore, we used an ultra-long GnRH agonist protocol, which is less common worldwide. In fact, we believe that the ultra-long protocol is effective, flexible, and more patient-friendly when combined with long-acting gonadotropin to reduce the injection times.

We acknowledged that the limitations of our studies included a small sample size and the retrospective nature of the second part of the study. In addition, our results did not take into account the condition of the endometrium and other clinical parameters such as ploidy status, fertilization rate, and live birth rate. A randomized prospective clinical trial with a larger sample size is needed to validate the preliminary results.

In conclusion, our results suggested that low-dose GH supplementation may improve oocyte and embryo yields, clinical pregnancy rate, and ongoing pregnancy rate in POR patients, especially in patients younger than 40 years old. The effect of low-dose GH in POR patients resulted in non-inferior clinical pregnancy outcomes compared with NRs.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study involving human participants was reviewed and approved by the Joint Institutional Review Board of Taipei Medical University (TMU-JIRB, approval number: N201505056) for retrospective analysis and clinical data reporting. TMU-JIRB waived the requirement for further specific ethics approval or written informed consent since all procedures were not altered from routine clinical protocols, in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

The present work was designed by C-RT. Data extraction and analysis were performed by Y-XL and M-SS. Patient recruitment was undertaken by C-RT. The initial manuscript draft was prepared by Y-XL and subsequently revised by Y-XL and C-RT. All authors approved the final submitted version.

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The Potential Role of Growth Hormone on the Endometrium in Assisted Reproductive Technology

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Growth hormone (GH) has been considered as an adjuvant treatment in human assisted reproductive technology (ART) for several years. Its action was largely attributed to an improvement of ovarian function and less emphasis was paid to its role in the uterus. However, there is increasing evidence that GH and its receptors are expressed and have actions in the endometrium and may play an important role in modifying endometrial receptivity. Thus, in this review, we firstly describe the existence of GH receptors in endometrium and then summarize the effects of GH on the endometrium in clinical situations and the underlying mechanisms of GH in the regulation of endometrial receptivity. Finally, we briefly review the potential risks of GH in ART and consider rationalized use of GH treatment in ART.

Keywords: growth hormone, endometrial receptivity, mechanisms, risk, ART

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INTRODUCTION

Growth hormone (GH), also called as somatotropin, is a protein of 191 amino acids with a three-dimensional structure that interacts with its receptor (1). The synthesis and secretion of GH is dynamically regulated by growth-hormone releasing hormone and somatostatin, which are both produced by the hypothalamus (2). In recent decades, GH has been considered to regulate many physiological functions including growth, metabolism, and reproduction (3, 4). Its therapeutic use in reproduction has been growing but remains controversial for its efficacy (4, 5). It has been used in assisted reproductive technologies (ART) in humans with an emphasis on potentially improved oocyte quality and pregnancy rates (6, 7). Little attention has been paid to the endometrium, despite the documented presence of GH receptors in this tissue (8). Our review of the literature indicates that study and application of GH for modulating the endometrium has been overlooked in understanding normal endometrial function in ART and reproduction generally. The endometrium undergoes changes via integrated interactions between the different uterine cell types and various growth factors and hormones (9). Several methods can be used to evaluate receptivity of the endometrium, including ultrasound, histology and molecular biomarkers (9, 10). Recent evidence has indicated that GH supplements may modulate endometrial receptivity and improve pregnancy outcomes in ART (10–13). Among women undergoing *in vitro* fertilization (IVF) treated with GH, changes of endometrial thickness (EMT) and endometrial perfusion have been described by ultrasound evaluation (11–14). The alteration of several biological markers of endometrial receptivity has also been detected with adjuvant administration of GH in animal models and cell-line studies (15–18). While short-term use of GH would not be expected to have

problems currently, it could potentially have side-effects on other diseases, especially active cancer and metabolic diseases (19).

We describe the data on evidence of GH action and its receptor in the endometrium. We then mainly focus on the current evidence for the influence of GH on endometrial receptivity. Finally, we look at the potential risks of GH in co-treatment in ART.

METHODS

A comprehensive search of the literature available in the PubMed, Web of Science, Embase, and CNKI was conducted using the following keywords, MeSH terms and phrases in combination with one another; "growth hormone," "somatotropin," "endometrium/uterine receptivity," "endometrial thickness," "endometrium perfusion," "endometrium cancer," "disease," "metabolism," "side effect/adverse event" "uterus/endometrium," "growth hormone knockout," "infertility," "reproduction" through July 2019. Both human studies and animal data were used.

GH AND ITS RELATED RECEPTOR IN ENDOMETRIUM

GH mediates its functions by binding to the GH receptor (GHR) (2). GHRs are most abundant in the liver (20), but also have been found in the reproductive system. GHRs have been reported in human granulosa cells and GH co-treatment in women receiving ART could regulate the expression of GHRs to improve pregnancy outcomes (7). The uterus also appears to be a site of both GH and GHR expression (2). GH has been detected in the cytoplasm of proliferating uterine epithelium cells in dogs (21) and also in human endometrial glandular cells during the mid and late luteal phases and in decidual tissue cells throughout pregnancy (22). GHRs can be found in uterine cells from various species including the mouse where localization of GHR mRNA in the endometrium, glands, stroma and myometrium have been described (23). GHR mRNA was also detected in the uterine epithelium, glands, vessels and placenta from bovine species (24) with biomolecular expressions including GHR and insulin-like growth factor-I (IGF-I) demonstrated in the uterus of dairy cows (25). In the pig, mRNA analyses demonstrated a high level of expression for endometrial somatotropin receptors (STR) (26). In women, GHR mRNA has been detected in the nuclei and cytoplasm of both human myometrial and leiomyoma cells (8). All these findings indicate a potential role for GH on the endometrium.

CLINICAL EVIDENCE OF GH ON ENDOMETRIAL RECEPTIVITY

Endometrial thickness (EMT) and uterine perfusion are important clinical indicators of endometrial receptivity in ultrasound studies (10). It has been suggested that ultrasonographic parameters including EMT and uterine perfusion can predict implantation potential in infertile patients undergoing embryo transfer (27). Although this is controversial (28), recent studies suggest a positive relationship between EMT and pregnancy outcome (29–32). Patients with positive pregnancy outcomes following IVF treatment had thicker endometrium readings on the day of hCG administration compared with those where a pregnancy did not result (29). The thicker the endometrium evaluated on the day of human chorionic gonadotropin administration, the higher the pregnancy rates reported following IVF (30, 31). EMT can also be measured on the day of oocyte retrieval and have been alleged to predict the endometrial receptivity during fresh IVF cycles (32). In general, EMT should exceed 8 mm as the threshold of endometrial receptivity in fresh embryo transfer cycles (33), although other studies suggest 10 mm of EMT may be better for a more stable implantation of embryos and minimization of pregnancy losses (34). Hence, increasing endometrial thickness and uterine perfusion might be beneficial goals for improving endometrial receptivity.

Two reports of women with panhypopituitarism causing either primary or secondary infertility who were treated with GH and gonadotropins are illustrative of the potential role for GH in fertility promotion (35, 36). After GH treatment, an improvement in their response to gonadotrophin stimulation was demonstrated with an acceptable endometrial growth and successful pregnancies ensued (35, 36). Standard infertile patients also show different endometrial changes and different pregnancy outcomes after adjuvant GH treatment (**Table 1**). For infertile women classified as poor responders, GH treatment has been promoted for improving the chances of pregnancy and live birth outcomes. Although no significant increases in implantation or clinical pregnancy rates are consistently demonstrated, there appears to be an increase of retrieved oocyte numbers and EMT (39, 42). A large scale retrospective clinical trial of infertile women classified as normal responders also had an increase in endometrial thickness in the older group (age $\geq$ 35 years) utilizing GH treatment and an improvement of implantation rate (IR) and clinical pregnancy rate (CPR) was claimed in the GH treatment group across all ages (45). An effect on weight-related infertility has also been seen with a significant improvement of EMT, IR and CPR in a group of infertile women who were overweight and obese (BMI $\geq$ 24 kg/m^2) (43, 45).

In patients with repeated implantation failure (RIF), a thicker endometrium on the day of hCG and an increase of IR, CPR and live birth rate (LBR) was found in a GH treated group, consistent with the previous results reported by others (11, 38). In another recent randomized clinical trial (RCT), the patients with RIF in an oocyte donation program also showed an increase of EMT, CPR, and LBR with GH supplements. Since the oocytes were donated by fertile women, further effects of GH on endometrium could be claimed (12).

In infertile women with poor endometrial development (EMT $<$ 7 mm), additional GH treatment is alleged to improve the EMT through uterine perfusion as well as the classical endometrial trilaminar pattern, although there was no significant alteration of pregnancy outcomes in this study (40). A meta-analysis including

TABLE 1 | The clinical evidence of GH on endometrial receptivity to improve pregnancy outcomes in ART.

Years	Study design	Objectives	Samples	Programme	Intervention	Outcomes	Effects	References
2007	Retrospective study	Infertile women with GH deficiency	20	IVF/ICSI	12 IU GH every third day, starting on the day of gonadotropin stimulation, till the administration of hCG	EMT FR	(–) ↑	(37)
2011	Prospective study	Infertile women with RIF	55	IVF-ET	4IU GH daily until the day of hCG administration	EMT IR/CPR	↑ ↑	(38)
2012	Randomized prospective study	Infertile women with poor responder	40	IVF-ET	4 IU GH from day 21 of previous cycle until the day of hCG injection	EMT IR/CPR Retrieved oocytes Obtained embryos FR	(–) (–) ↑ ↑ ↑	(39)
2013	Prospective study	Infertile women with endometrial dysplasia	32	FET	4IU GH daily until the day of hCG administration	EMT Trilaminar pattern Uterine perfusion CPR	↑ ↑ ↑ (–)	(40)
2015	Retrospective study	Infertile women with thin endometrium (EMT<7 mm)	35	FET	4IU GH daily, starting from the 3–5th day of menstrual cycle, until the day of progesterone administration	EMT CPR	↑ ↑	(41)
2016	Parallel randomized, open-label study	Infertile women with poor responder	68	IVF/ICSI	2.5 mg(7.5IU) GH daily, starting on day 6 of hMG stimulation until the day of hCG triggering	EMT IR/CPR	↑ (–)	(42)
2016	Prospective study	Infertile women with overweight/obesity	33	IVF-ET	4.5 IU GH daily, starting from the day of hMG administration till the day of hCG	EMT IR/CPR	↑ ↑	(43)
			34	IVF-ET	4.5 IU GH every alternate day, starting from the day of hMG administration till the day of hCG	EMT IR/CPR	↑ ↑	(43)
2016	Prospective study	Infertile women with thin endometrium (EMT<8 mm)	5	FET	4-5 times of GH intrauterine perfusion (6IU/0.5 ml 0.9% saline) on the ninth to twelfth day of hormone replacment cycle	EMT	↑	(44)
2016	Prospective study	Infertile women	77	FET	4 IU of rhGH daily from day 3 of the menstrual cycle until the day of progesterone injection	Serum E2 IGF-I VEGF EMT Perfusion of the uterine endometrial arcuate artery IR/CPR/LBR	↑ ↑ ↑ ↑ ↑ ↑	(13)
			76	FET	4 IU of rhGH daily from day 8 of HRT until the day of progesterone injection	EMT IR/CPR	(–) (–)	(13)
2016	Retrospective clinical trial	Infertile women with a normal ovarian response to controlled ovarian hyperstimulation (COH)	556	IVF-ET	4.5 IU of GH daily, starting from the initial day of gonadotropin treatment and lasting for 5 days	EMT in older group (≥35 years old) IR/CPR	↑ ↑	(45)
2018	Randomized controlled trial	Infertile women with RIF	35	IVF/ICSI	NM	EMT IR/CPR	↑ ↑	(12)
2018	Prospective study	Infertile women with RIF	22	IVF-ET	4 IU GH daily until the day of hCG administration	EMT IR/CPR/LBR	↑ ↑	(11)
2018	Randomized controlled trial	Infertile women with thin endometrium (EMT<8 mm)	63	NM	4IU GH daily for 10 days	EMT Uterine perfusion CPR	↑ ↑ ↑	(46)

(Continued)

TABLE 1 | Continued

Years	Study design	Objectives	Samples	Programme	Intervention	Outcomes	Effects	References
2018	Prospective study	Infertile women with thin endometrium (EMT < 7 mm)	40	IVF/ICSI	5 IU GH daily, starting at the first 4 days till the 18th day of the cycle	EMT IR/CPR	↑ ↑	(14)
2019	Retrospective study	Infertile women with thin endometrium (EMT < 8 mm)	184	FET	4.5 IU GH every alternate day, starting from the day of progesterone administration till the day of ET	EMT IR/CPR	(–) ↑	(47)

↑ = increase; (–) = No significant change.
Ref, Reference; NM, Not mentioned.
RIF, Repeated implantation failure; IVF, In vitro fertilization; ICSI, Intracytoplasmic sperm injection; FET, frozen-thawed embryo transfer; ET, embryo transfer; rhGH, recombinant human GH; hCG, human chorionic gonadotropin; hMG, human menopausal gonadotropin; EMT, endometrial thickness; FR, Fertilization rate; IR, Implantation rate; CPR, Clinical pregnancy rate; LBR, Live birth rate.

four RCTs demonstrated an enhancement effect of GH on EMT in infertile women with poor endometrial development (EMT < 6 mm or non-trilaminar type endometrium) [OR = 10.62, 95% CI (2.97, 38.00)] (48). Other studies also demonstrated that EMT with GH treatment was significantly increased on day 3 (the 18th day of cycle) with subsequent increased IR and CPR (14) and an increased EMT was also detected in five patients with thin endometrium (EMT < 8 mm) after intrauterine perfusion of GH or parenteral injection of GH (41, 44, 46). These observations are not confirmed by others however (13, 37, 39, 47). Different patient selection, doses, starting time as well as the different measurements and interpretations may be the reasons resulting in the varied outcomes of GH treatment (**Table 1**). This is illustrated in a recent prospective study where the same dose of recombinant human GH (rhGH) was applied to infertile women but with different starting times resulting in quite different clinical outcomes (13). Those women who started with rhGH treatment earlier in the cycle had a significant increase of EMT, perfusion of the uterine artery index, IR, CPR, and LBR as well as estradiol, IGF-I and vascular endothelial growth factor (VEGF) on the day of embryo transfer (13).

POTENTIAL MECHANISMS OF GH ON ENDOMETRIAL RECEPTIVITY

The mechanisms of GH effects on the endometrium to improve the EMT and uterine perfusion and IVF outcomes are still unclear. Currently, several molecules, including IGF, leukemia inhibitory factors (LIF), integrins (Itg), homeobox-containing transcription factors-HOX family genes, etc. contribute to the molecular basis of regulating endometrial receptivity while some molecules closely related to the implantation process have been demonstrated to be involved in the potential mechanisms of GH effects on endometrium (9, 49) (**Table 2**).

Animal Models

Increased concentrations of cytosolic estrogen receptor but not the concentration of progesterone receptor was found in the rabbit uterus after GH treatment, indicating a potential estrogen mediated function (50). Consistent with this, an increase in the concentration of estrogen receptor in the guinea-pig uterus after treating with GH has also been demonstrated (51). Research in the ovine uterus indicates that GH could regulate endometrial gland proliferation via interferon tau (52) and could alter the endometrial gene expression related to maintenance of pregnancy. GH may increase the expression of oxytocin receptor, progesterone receptor mRNA, and the mRNA of estrogen receptor α in non-lactating cows (53) with an increased pregnancy rate in lactating cows after injection of GH at the initiation of timed artificial insemination following a synchronized ovulation protocol (54). Knockout of the GHR in mice leads to a negative impact on reproduction with fewer uterine implantation sites during early pregnancy (55).

TABLE 2 | The effects of GH on endometrial receptivity via molecular biomarkers.

Years	Species	Samples	Intervention	Outcomes	Effects	References
Animal models						
2005	Porcine	33	6 mg of porcine somatotropin	Endometrial STR, IGF-I, IGF-II, IGFBP2	↑	(25)
2006	Mouse	25	0 15 IU GH/100 g estrous cycle	LIF, Itgαvβ3, MMP-9	↑	(17)
2007	Mouse	25	1.5 m IU GH/g proestrous stage	VEGF, LIF, MMP-9, TIMP-1	↑	(16)
2012	SD rats	25	0 15 IU GH/100 g proestrous stage	Itgαvβ3, OPN	↑	(18)
***In vitro* cell studies**						
Years	**Cell types**		**Intervention**	**Outcomes**	**Effects**	**References**
2019	RL95-2 cells		Cultured for 48 h in the presence of GH (10 nM)	Cell proliferation; Activates cell cycle; VEGF, ItgB3 and IGF-I	↑	(13)
				Itgαv, LIF, EGF, HOXA10, and SPP1	(–)	(14)
2010	Endometrial stromal cells		4 ng/ml GH; 5 ng/ml IGF-I	Cell proliferation	↑	(15)
	Decidual cells		5 ng/ml IGF-I	Cell proliferation	↑	(15)

↑ = increase; (-) = No significant change.

STR, Somatotropin receptor; IGF(BP), Insulin-like growth factor (binding protein); LIF, Leukemia inhibitory factors; Itg, Integrin; MMP, Matrix metalloproteinase; VEGF, Vascular endothelial growth factor; TIMP, Tissue inhibitor of metalloproteinase; OPN, Osteopontin; SD rats, Sprague Dawley rats; EGF, Epidermal growth factor; HOXA, HOX family; SPP1, Secreted phosphoprotein.

GH-mediated increase in uterine IGF-I levels may be the mechanism underlying the increase in endometrial thickness (56). Exogenous porcine GH elevates the expression of endometrial STR, IGF-I mRNA, IGF-II mRNA, and IGFBP2 mRNA in the uterus, supporting the role of the so-called GH/IGF axis in the uterus (25, 56). Both IGF-I and IGF-II can be detected in endometrial stroma, but have different roles (2, 57). IGF-II is more closely related to endometrial differentiation (57), while IGF-I is a potential mediator of the mitogenic effects of estrogen on the uterus, so-called oestromedin (56). Other studies in the mouse also indicate an alteration of other molecular biomarkers of endometrial receptivity after treating GH (16, 17). GH-treated mice show a significant increase of leukemia inhibitory factors (LIF), integrin alpha v beta 3(Itgαvβ3) and matrix metalloproteinase (MMP)-9 in the endometrium, molecules which have been implicated in implantation (16, 17). Exogenous GH supplementation in Sprague Dawley (SD) rats may also increase osteopontin (OPN) and Itgαvβ3 expression with an associated improvement in endometrial receptivity (18).

In vitro Studies

Addition of hGH to the cultured endometrial and decidual cells increases the proliferation of endometrial and decidual cells, when these cells were harvested and separated from the human endometrial pieces and decidual tissue, respectively (15). When transfected to the human endometrial cell line RL95-2, there is an enhancement of cell proliferation, survival and invasion (58) and increased cell proliferation and expression of VEGF, Itgβ3, and IGF-I (14). Janus kinase (JAK) 2 inhibitor AG490 addition with GH suppresses VEGF, Itgβ3, and IGF-I expression in RL95-2 cells, indicating that GH might regulate the expression of these factors via the JAK2 pathway. However, no change in expression of LIF, Itgαv, Hox family gene (HOXA10) and SPP1 could be found in RL95-2 cells after treatment with GH (14). Therefore, the effects of GH on endometrial receptivity-related molecules *in vitro* remains to be elucidated.

THE POTENTIAL RISKS OF GH TREATMENT IN ART

GH may play a pathological role in body systems in view of the fact that it may have a potential of being an oncogene (59). Transplantation of GH transfected-RL95-2 cells suspension to BALBc nu/nu mice led to a larger tumor size and a more aggressive progression of endometrial carcinoma (58). When RL95-2 cells cultured with the GH receptor antagonist (pegvisomant) were inoculated into immunodeficient NIH-III mice and continuously treated the mice with antagonist for 16 weeks, a delayed tumor growth rate and decreased IGF serum level could also be found (60). A systematic review concluded that long-term GH treatment actually had a positive effect on reducing cardiovascular disease, stroke, and fractures, without a simultaneous increase in malignancy risk (61). While there are few indications of problems, the long-term safety of GH for the cancer risk, metabolic disorder and other unforeseen adverse events should be under constant surveillance (62).

Although metabolic sequelae improve with GH in GH deficient patients (63), some advocate that addition of GH in patients with diabetes mellitus should be cautioned due to its potential negative effects on insulin resistance and glucose tolerance (19, 64). GH can also result in significant metabolic changes by elevating cholesterol and disturbing the renin-angiotensin mechanism (65). Therefore, in consideration of the potential risks, personalized comprehensive assessment, and professional guidance in usage and dosage is required before deciding to use GH in ART.

CONCLUSION

Clinical evidence for efficacy of GH in improving reproductive function remains controversial. Generally, the majority of studies show positive effects of GH on endometrial receptivity, but there is no general agreement about the dosage and usage of GH in ART and there are few useful RCTs (**Table 1**). Hence, more evidence is still required to determine the purported value of GH treatment in ART and provide more specific guidance in the clinical setting.

Even if clinical evidence currently encourages the view that GH might be helpful for endometrial receptivity, the mechanisms are still not known. The studies of molecular biomarkers included in this review are few (**Table 2**), but also may provide some foundations for future exploration of the mechanisms of GH in the endometrium. As GH is administered systemically during ART, it is difficult to separate the effect on GHR in the ovary from that in the endometrium and if GH alters receptor action in the ovary, it could potentially have an effect on the endometrium receptors too.

The risk of GH used in ART should be noticed but not overstated. There is a relationship between autocrine GH and endometrial cancer, but further studies of the mechanisms under this phenomenon and the confirmation of increased diseases risk of exogenous GH are still needed. In general, further explorations of the mechanisms underlying the effects of GH on endometrium should spread some light on our basic knowledge and clinical actions.

AUTHOR CONTRIBUTIONS

F-TL and RL were responsible for writing the first draft. RL, ZW, JY, and RN performed the critical revisions. All authors listed did contributed to the writing and review of the manuscript.

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The Effect of GH Administration on Oocyte and Zygote Quality in Young Women With Repeated Implantation Failure After IVF

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Growth hormone (GH) has been shown to improve implantation and live birth rates in women of >40 years of age treated by *in vitro* fertilization (IVF). This effect was initially attributed to a GH effect on oocyte quality, but later studies showed that GH can also improve uterine receptivity for embryo implantation. As to younger women with previous failures of embryo implantation after IVF, data reported in the literature are ambiguous. This retrospective study focused on this latter category of women, comparing the numbers and morphological appearance of oocytes recovered from women with two previous IVF failures, aged between 30 and 39 years and treated with GH, with a comparable group of women without GH treatment. These results were complemented with the analysis of morphological markers of zygote and embryo quality and IVF clinical outcomes in both groups. The oocytes, zygotes and embryos from women treated with GH showed better morphological scores, and their uterine transfer resulted in more implantations, pregnancies and live births, as compared with the untreated group. It is concluded that the improvement of IVF outcomes in women with previous repeated IVF failures by exogenous GH administration is, at least partly, related to an increase in oocyte developmental potential. The statistically evident improvement of oocyte and embryo quality is the main finding of this study. Its weakness is its retrospective nature.

Keywords: oocyte quality, embryo quality, implantation rate, live birth rate, growth hormome

INTRODUCTION

Exogenous growth hormone (GH) administration has been introduced to protocols of ovarian stimulation for *in vitro* fertilization (IVF) since the late 1980s and shown to improve IVF clinical outcomes (1–7), in agreement with observations on a positive relationship between GH concentration in follicular fluid aspirated from ovaries of patients treated by IVF and the treatment outcomes (8, 9). However, in spite of these encouraging initial data, some subsequent studies failed to find an improvement of IVF clinical outcomes after the inclusion of GH in the ovarian stimulation protocol (10, 11). These data suggest that GH treatment cannot improve IVF outcome in all patients with poor response to ovarian stimulation and open the question of how to identify patients who can benefit from this treatment.

There is solid evidence indicating that GH co-treatment during ovarian stimulation can enhance IVF outcomes in women aged >40 years (12, 13), but also in some younger women with previous repeated IVF failures (14), low response to high-dose stimulation (15, 16) and poor oocyte and embryo quality (14). Some studies have suggested an effect of GH on oocyte quality rather than quantity, through an improvement of cytoplasmic maturation with consequent reduction of aneuploidy caused by errors in the first and the second meiotic divisions (12, 14), while others also showed an effect on the number of retrievable oocytes, mediated by an increase in FSH- LH- and bone morphogenetic protein (BMP)- receptor density, as well as the density of its own receptors in granulosa cells, by GH treatment (13). Interestingly, a recent study reported an increase in the number of total retrieved, mature and fertilized oocytes, available embryos and high-quality embryos in all women with poor ovarian response treated with GH, independently of their age, but a significant increase in the implantation and pregnancy rate was found only in the older patients (17).

Moreover, the beneficial effects of GH administration on IVF outcomes, demonstrated in some patients, may not be caused solely by the hormone effect on the ovarian function. In fact, recent data have shown that the treatment with GH can also promote embryo implantation by improving uterine receptivity. This was demonstrated by two studies in which an effect of GH on oocyte quality could be excluded. Both studies used GH during the preparation of women for the transfer of embryos resulting from oocytes obtained in ovarian stimulation cycles not including GH administration. One dealt with transfers of the patients' own cryopreserved embryos resulting from a previous ovarian stimulation (18), and the other with transfers of fresh embryos from donated oocytes in patients with previous unexplained oocyte donation failure (19). Another study suggested that GH can both improve embryo quality and increase endometrial thickness in patients undergoing IVF, the former effect being more pronounced in women of <35 years of age and the latter in the older ones (17, 20). Altogether, the published data suggest that GH administration during ovarian stimulation can improve IVF outcomes in some, but not all, cases. It is not clear whether this effect is mainly due to the action of the hormone on oocyte quality or uterine receptivity, and how it is related to the patient's age. While the main cause of IVF failure in older women is supposed to be related with oocyte aneuploidy, mainly due to premature loss of centromeric cohesion between sister chromatids (21), and GH appears to alleviate this condition (12, 14), this may not be the case in younger women with poor response to ovarian stimulation treatments (22) in whom the mechanism of GH effect of IVF outcomes is even less clear.

This study was undertaken to evaluate the effects of GH administration during ovarian stimulation on the number of oocytes retrieved as well as on different morphological markers of oocyte and embryo quality and on IVF outcomes in young women with previous IVF failures.

Abbreviations: GH, Growth hormone; IVF, *In vitro* fertilization; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; BMP, Bone morphogenetic protein; ICSI, Intracytoplasmic sperm injection; IMSI, Intracytoplasmic morphologically-selected sperm injection; GnRH, Gonadotropin releasing hormone; HMG, Human menopausal gonadotropin; HCG, Human chorionic gonadotropin; SD, Standard deviation.

TABLE 1 | Baseline characteristics of women ($n = 98$) treated ($n = 52$) and untreated ($n = 46$) with GH.

Treatment	Age (year)	BMI (kg/m^2)	Infertility duration (year)	AFC (*n*)	Serum AMH (ng/ml)
Without GH	34.5 ± 4.9	21.9 ± 4.2	3.9 ± 1.8	6.8 ± 4.1	2.3 ± 1.4
With GH	34.8 ± 4.1	22.2 ± 4.3	4.1 ± 2.1	6.4 ± 4.0	2.2 ± 1.5
P-value	>0.05	>0.05	>0.05	>0.05	>0.05

Values are mean ± SD (%).

TABLE 2 | Effect of GH treatment during ovarian stimulation on oocyte quality.

Treatment	Metaphase II (MII) oocytes retrieved per patient[a]				
	Total MII oocytes	Type A	Type B	Type C	Type D
Without GH	6.5 ± 3.2 (100)	2.9 ± 2.7 (45)	1.6 ±1.4 (25)	1.0 ± 1.3 (15)	1.0 ± 1.3 (15)
With GH	6.9 ± 2.9 (100)	4.1 ± 2.9 (59)	1.7 ± 1.7 (25)	0.8 ± 0.9 (12)	0.3 ± 0.6 (4)
P value	>0.05	<0.05	>0.05	>0.05	<0.01

[a] *Values are mean ± SD (%).*

RESULTS

In spite of the retrospective character of this study (see Materials and Methods), the patients treated and those not treated with GH were similar as to their age, duration of infertility, and basic parameters of ovarian function (**Table 1**).

With the same protocol of ovarian stimulation, taking into account the individual condition of each patient and adjusted during the stimulation according the patient's response as described previously (23, 24), there was no difference in the total number of oocytes (**Table 1**) and of mature (metaphase II) oocytes (**Table 2**) retrieved from women co-stimulated with GH as compared with those in whom GH was not used. By contrast the number and percentage of oocytes with the best cumulative morphological quality score (Type A, **Figure 1**.1) was significantly higher, and those of oocytes with the worst score (Type D, **Figure 1**.4) was significantly lower in patients treated with GH as compared with the untreated patients (**Table 2**). As to the number and percentage of oocytes with the intermediate cumulative quality scores (Types B, **Figure 1**.2 and C, **Figure 1**.3), there was no difference between the two groups of patients (**Table 2**). No significant difference in endometrial thickness ($P > 0.05$) was detected between the protocols that included GH (9.0 ± 1.3 mm) and those that did not (8.8 ± 1.2 mm).

Patients treated with GH had significantly more total zygotes and good-quality zygotes, according to the evaluation of pronuclear morphology (**Table 3**), more total cleaving embryos

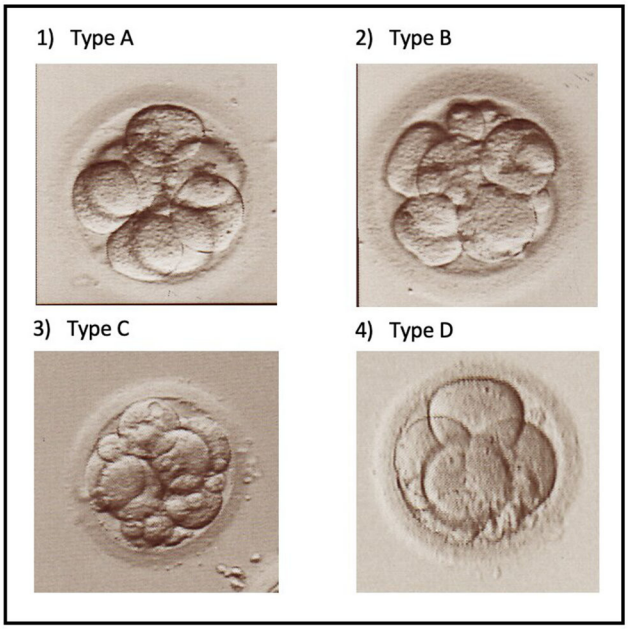

FIGURE 1 | Micrographs showing different types of cleaving embryos on day 3 after ICSI. (1) Type A embryo with adequate number of cells of equal size (8) and only a few cell fragments. (2) Type B embryo with adequate number of cells (8) but some of unequal size, and a larger volume occupied by cell fragments. (3) Type C embryo with a lower number of cells (6) and numerous cell fragments. (4) Type D embryo apparently blocked at the 4-cell stage and some cell fragments.

TABLE 3 | Effect of GH treatment during ovarian stimulation on zygote quality.

Treatment	Zygotes with normal and abnormal pronuclear pattern achieved per patient[a]		
	Total	Normal pattern	Abnormal pattern
Without GH	4.6 ± 2.5 (100)	0.6 ± 0.8 (13)	4.0 ± 2.0 (87)
With GH	5.8 ± 2.5 (100)	1.4 ± 1.2 (24)	4.4 ± 2.4 (76)
P-value	<0.05	<0.01	>0.05

[a] *Values are mean ± SD (%).*

TABLE 4 | Effect of GH treatment during ovarian stimulation on the quality of embryos achieved.

Treatment	Embryos achieved per patient[a]				
	Total	Type A	Type B	Type C	Type D
Without GH	4.7 ± 2.5 (100)	1.4 ± 1.2 (30)	1.7 ± 1.6 (36)	1.0 ± 0.8 (21)	0.6 ± 0.8 (13)
With GH	5.9 ± 2.4 (100)	3.2 ± 2.1 (54)	1.9 ± 1.4 (32)	0.6 ± 0.8 (10)	0.0
P-value	<0.05	<0.01	>0.05	<0.05	<0.05

[a] *Values are mean ± SD (%).*

TABLE 5 | Effect of GH treatment during ovarian stimulation on the quality of embryos transferred.

Treatment	Embryos transferred per patient[a]			
	Total	Type A	Type B	Type C
Without GH	2.4 ± 0.6 (100)	1.0 ± 1.1 (42)	0.9 ± 0.7 (37)	0.5 ± 0.7 (21)
With GH	2.2 ± 0.6 (100)	1.8 ± 0.8 (82)	0.3 ± 0.6 (14)	0.1 ± 0.1 (4)
P-value	>0.05	<0.05	<0.01	<0.01

[a] *Values are mean ± SD (%).*

TABLE 6 | Effect of GH treatment on clinical pregnancy and delivery rate.

Treatment	Embryo transfers	Clinical pregnancies	Deliveries	Pregnancy rate	Delivery rate
Without GH	46	5	3	10.9%	6.5%
With GH	52	22	18	42.3%	34.6%
P-value				<0.01	<0.01

TABLE 7 | Effect of GH treatment on clinical implantation and birth rate.

Treatment	Embryo transferred	Gestational sacs with heartbeat	Babies born	Clinical implantation rate	Birth rate
Without GH	110	5	3	4.5%	2.7%
With GH	104	22	18	21.2%	17.3%
P-value				<0.01	<0.01

and those with the highest cumulative morphological quality score (Type A) and less embryos with the lowest scores (Types C and D) as compared with the untreated patients (**Table 4**). Like the oocyte quality scores (**Table 2**), there was no significant difference in the number and percentage of embryos with intermediate quality score (Type B) between both groups (**Table 4**).

Similar numbers of embryos were transferred in patients treated and in those untreated with GH, but the patients of the former group received more high-quality embryos as compared with those of the latter (**Table 5**). More patients treated with GH became pregnant after embryo transfer, and developed more gestational sacs, as compared with the untreated patient group (**Table 6**). Consequently, both pregnancy rate and implantation rate were significantly improved by GH administration (**Table 6**).

Eighteen healthy babies were born in patients treated with GH, as opposed to only 3 in the untreated patient group, marking a significant difference in both the delivery rate (**Table 6**) and birth rate (**Table 7**) in favor of the GH-treated patient group.

No complications were observed in either group of patients during and after ovarian stimulation.

DISCUSSION

The present data show that, independently of eventual effect on uterine receptivity, GH has a clear beneficial influence on the quantity and morphological quality of oocytes zygotes and

cleaving embryos when administered to young women with previous IVF failures. These improvements are accompanied by a significant increase in the clinical pregnancy, delivery, implantation and birth rates in this group of patients. No multiple pregnancy was established in either of the two groups, which is somewhat surprising especially in the GH group. Though this might be a matter of chance, it is also possible that some of the patients had other predisposing factors for implantation failure, not resolvable by GH treatment.

Unlike the study by Jin et al. (17), the number of mature (metaphase II) oocytes retrieved in patients treated and those untreated with GH was similar. However, in agreement with those previous observations (17), there were more good-quality oocytes, fertilized oocytes and good-quality zygotes and embryos in the GH group. GH administration was shown to enhance FSH- LH- and bone morphogenetic protein (BMP)- receptor density in ovarian follicular granulosa cells (13). This effect may lead to an increase in the number of retrievable oocytes in women with poor ovarian response (13, 17), whereas it may improve the quality rather than the quantity of oocytes recovered from women with basically normal ovarian response. This kind of patients was prevalent in the present study. This hypothesis is further substantiated by the present observation that GH administration in this group of patients did not only improve the morphological quality of the oocytes obtained, but it also increased the total number of fertilized oocytes (zygotes), that of normal zygotes and that of high-quality embryos, as judged by their morphological appearance. Among these characteristics, that of the zygote quality seems to be of particular importance, since it was previously shown that zygote pronuclear morphology is related not only with IVF clinical outcomes (25–28) and the rate of embryo development to the blastocyst stage (27), but also with the normal ploidy of the resulting blastocysts (29–31). When used in combination with further evaluation of embryo morphology during subsequent stages of preimplantation development, the prognostic value of zygote morphology, as to the probability of establishing a normal pregnancy, was further enhanced (32, 33).

This study showed a significant improvement of both zygote and cleaving embryo morphology by the administration of GH during ovarian stimulation. This suggests that the improvement of oocyte quality is an important mechanism of action of GH responsible for the improvement of pregnancy, implantation, delivery and live birth rates in young women with previous IVF failures. If confirmed, the up-regulation of granulosa cell receptors by GH may be involved in oocyte cytoplasmic maturation which, in its turn, may stabilize the function of cohesin and other key proteins involved in the correct function of the meiotic spindle during the final phases of oocyte nuclear maturation. An effect of GH on embryo implantation may have also acted as an independent factor in some of these cases, but it appears to be marginal as compared with the effect on oocyte quality, in agreement with the previous observation of a relatively low prevalence of cases with repeated implantation failures after oocyte donation resolved by GH administration to oocyte recipients (19).

The mechanism through which exogenous GH can improve oocyte quality in young women remains to be elucidated. It may be related to the previously reported increase in the density of receptors for FSH, LH, BMP receptor 1B, as well as its own receptor (13). However, the above observations were obtained with an older patient population as compared with that involved in our study. It remains to be determined whether GH produces similar effects in younger women with previous IVF failures, supposedly related to poor oocyte quality. It remains to be determined whether the beneficial effects of GH on oocyte quality are mainly mediated by a direct action through its own receptors or by an increase in the secretion of IGF-1. Studies are in progress to address these questions in order to characterize better those women who are likely to benefit from GH co-stimulation to improve IVF outcomes. In addition to the effect on oocyte quality, improvement of uterine receptivity (19) may also have contributed to the positive effects of GH on embryo implantation in some patients, although no difference in endometrial thickness was found between patients who were treated with GH and those who were not. However, endometrial receptivity is not necessarily reflected by endometrial thickness, and the design of this study does not allow to discriminate between these two mechanisms. This would only be possible with an oocyte donation model.

It also remains to be determined why GH administration has more effect in some young women than in others. While this paper was under review, we have addressed specifically this question, with another group of patients. We found that some young women have their "GH-age," determined indirectly by measuring their serum IGF-1 concentrations (GH is too fluctuating to give a reliable result) up to 20 years above their chronological age (34). This was not done in the women included in the present study. It seems that externally administered GH has less effect in young women with normal intrinsic GH production (34).

MATERIALS AND METHODS

Study Design, Participants, and Their Allocation to Groups

This retrospective study was approved by the ethical committee of our clinic. All procedures performed in this study were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments. All participants signed an informed consent. The study involved 98 women, aged between 30 and 39 years, and having undergone at least 2 previous unsuccessful IVF attempts in spite of generating acceptable numbers of oocytes and embryos. They were considered for a new treatment attempt at the MARGen Clinic in the period between January 2014 and December 2017. Fifty-two of these women were treated with GH during ovarian stimulation, whereas the other 46 were not. The patient allocation to each of the two groups was based on the couples' own decision after having received exhaustive information concerning the current knowledge about the use of GH in their situation.

In fact, our ethical committee discouraged a randomized controlled trial because, in view of our previous results, the deliberate allocation of patients to the control group may cause harm. Thus, all the pros and cons, as well as the lack of solid evidence in favor of GH were thoroughly discussed with each couple. The decision was then taken by the couples, not by the medical staff.

It was explained that GH has been shown clearly to improve IVF outcomes in older women, but its benefits for younger women undergoing IVF treatment is controversial. Since the short treatment with GH has no side-effects on the patient's health, the patients' decision as to the use of this treatment was sometimes motivated by its cost. Some patients also preferred not to be included in the GH group because of concern about potentially useless "overmedication," adding more daily injections to the already quite complex ovarian stimulation protocol. In spite of the absence of any artificial "matching," patients who decided to be included in the GH group had similar baseline characteristics as compared to those who preferred the standard ovarian stimulation protocol (**Table 1**). But for the exclusion of GH administration from the ovarian stimulation protocol, these patients were treated exactly as those of the GH group. Only couples with normal basic sperm parameters and normal percentage of spermatozoa with DNA fragmentation (35) were included.

Assisted Reproductive Technologies (ART)

IVF was performed by intracytoplasmic sperm injection (ICSI) in all patients involved in this study, after ovarian stimulation using a GnRH antagonist protocol. Details of both the clinical and the laboratory protocols used were published in detail previously (19). Briefly, patients were treated by daily injections of recombinant follicle-stimulating hormone (FSH) (Puregon or Gonal F) and human menopausal gonadotropin (HMG) (Menopur), started between the second and the fourth day following the beginning of menstrual bleeding. The initial doses of FSH and HMG were determined according to the markers of the patients' ovarian reserve, antral follicle count and serum concentration of LH on the day preceding the beginning of stimulation. If serum LH level before the beginning of ovarian stimulation was below 2 IU/L, HMG was added to FSH. If the small antral follicle count in both ovaries was equal to or higher than 10 and the serum LH concentration was between 1 and 2 IU/l, the usual daily dose of HMG during the first 4 days of stimulation was 75 IU. When there were <10 small antral follicles in both ovaries, the usual dose of HMG was 150 IU. The HMG treatment was always accompanied by FSH whose dose was adapted according to the patient's basal serum anti-Mullerian hormone concentration. The following ultrasound examinations, as well as the determinations of serum estradiol and LH concentrations were done on the 5th day of stimulation and then every other day until the administration of ovulation trigger. The respective doses of FSH and HMG administered were adapted, in a flexible manner, according to the results of each of these examinations, in the same way as described for the long GnRH agonist-controlled ovarian stimulation protocol (19). Briefly, FSH dose was basically determined according to serum estradiol concentration and the number and size of antral follicles. If serum LH concentration tended to decrease, especially after the onset of GnRH antagonist treatment, the growth of all follicles was slow, and no tendency for dominance was observed, higher doses of HMG (75–150 IU) were maintained. If, on the other hand, serum LH concentrations increased, follicular growth was rapid and some follicles grew more rapidly than others, HMG was maintained at minimal doses or even withdrawn, finishing the whole ovarian stimulation procedure with FSH alone. If different, and sometimes opposite, tendencies in all these parameters were observed, the clinician took the decision after pondering the advantages and disadvantages of different FSH-to-HMG dose ratios, taking into consideration the history and the complete clinical picture of each case. Ovulation was triggered by subcutaneous injection of 250 μg recombinant human chorionic gonadotropin (HCG; Ovitrelle) when at least two follicles reached the size of 17–18 mm. Ovarian puncture for oocyte recovery was performed 36.5 h after the HCG injection.

In spite of the fact that all male partners had normal sperm parameters, the high-magnification ICSI, also called intracytoplasmic morphologically selected sperm injection (IMSI) was used in all cases by precaution, taking into account the history of the patients' previous IVF failures, as described previously (36). All embryos were transferred on Day 3 after ICSI. At least one embryo of acceptable quality (cumulative scores A, B or C, excluding score D) was available for transfer in all cases. Luteal phase was supported with intravaginal micronized progesterone, beginning on the day of oocyte recovery, at daily doses ranging between 200 and 600 mg, according to serum progesterone concentration, determined on the day of embryo transfer and then every 7 days during the first month after the transfer.

Protocol of GH Administration

A total dose of 10 mg GH (Nutropin) was administered in 10 daily doses of 1 mg, starting on the first day of ovarian stimulation. When the ovarian stimulation was shorter than 10 days, the rest of the total 10-mg dose was administered on the day following the application of ovulation trigger. This short GH administration protocol was based on our previous work (12) aimed at improving oocyte quality rather than quantity. In fact, the women included in this study were relatively young and yielded sufficient numbers of oocytes in their previous unsuccessful treatment attempts.

Evaluation of Oocyte, Zygote, and Embryo Quality

Oocyte, zygote and embryo quality were evaluated by microscopical examination using an inverted microscope (Olympus IX71) equipped with Hofman modulation contrast optics. Oocytes were attributed one of the four cumulative scores, from A (the best one) to D (the worst one), taking into account the oocyte shape, cytoplasmic granularity, the presence of intracytoplasmic vacuoles, the form of the zona pellucida and the perivitelline space, and the size and morphology of the first polar body (37). Zygotes and cleaving embryos (**Figure 1**) were scored as described previously (25–28, 32). Briefly, cleaving

embryos were scored according to the number of cells, the shape and regularity of the cells, and the volume occupied by anucleate fragments (**Figure 1**).

Statistical Analysis

Analysis was performed using SPSS version 16.0 for Microsoft Office (SPSS Inc., Chicago, IL, USA). The data were expressed as mean ± SD for quantitative variables or percentage (%) of or qualitative ones. Data between the two groups were analyzed by Student's *t*-test for quantitative variables and X2 tests for qualitative ones. All tests were two-tailed and a $P < 0.05$ was considered statistically significant.

CONCLUSION

Independently of eventual contribution to an improvement of uterine receptivity, GH administration during ovarian stimulation of young women with previous IVF failures was clearly shown to improve IVF outcomes by increasing oocyte, zygote and embryo quality.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Foundation MARGen Mendoza Tesarik. Granada, Spain. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RM-T and JT: conceptualization, validation, formal analysis, and writing-review and editing and writing original draft preparation. RM-T, MG-L, and CC-L: laboratory work and validation JT: clinical evaluation. RM-T: software. All authors contributed to the article and approved the submitted version.

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