DIMINISHED OVARIAN RESERVE & POOR OVARIAN RESPONSE: DIAGNOSTIC AND THERAPEUTIC MANAGEMENT

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DIMINISHED OVARIAN RESERVE & POOR OVARIAN RESPONSE: DIAGNOSTIC AND THERAPEUTIC MANAGEMENT

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EDITORIAL
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Editorial: Diminished Ovarian Reserve and Poor Ovarian Response: Diagnostic and Therapeutic Management

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Keywords: diminished ovarian reserve (DOR), ovarian stimulation and response, IVF (in vitro fertilization), ART (assisted reproductive technology), poor ovarian response (POR)

Editorial on the Research Topic

Diminished Ovarian Reserve & Poor Ovarian Response: Diagnostic and Therapeutic Management

Over the last decades there has been a steep increase in the demand for ART treatment and the main reason for this is the advanced age of the couple trying to conceive. Another important aspect of the last years is the increased incidence of malignancies in young-adults and the consequent fertility preservation of cancer survivors. In contrast to what people are inclined to think, *in vitro* fertilization (IVF) treatments cannot fully compensate for age-dependent loss of fertility, as the success rate of any fertility techniques directly depend on maternal age (Mills et al., 2011). In fact, advanced maternal age (Wallace and Kelsey, 2010), and iatrogenic (ovarian surgery or gonadotoxic therapies) (Dewailly et al., 2014) or non-iatrogenic conditions (for instance the presence of genetic polymorphism at the levels of gonadotropin receptors) can reduce the ovarian reserve. Independently of the cause of diminished ovarian reserve (DOR), up to 1/3 of these patients experience a poor ovarian response (POR) to ovarian stimulation (OS) leading to cycle cancellation and a reduced chance of a live birth (Polyzos et al., 2012; Polyzos et al., 2014; La Marca et al., 2015; Polyzos and Popovic-Todorovic, 2020). The first consensus on the definition of POR (Ferraretti et al., 2011), the Bologna criteria, has been the first time this population was clinically defined; however, the most important limitation was the heterogeneity of the population included in the definition, given by grouping women with different biological characteristics and therefore prognosis (Polyzos and Drakopoulos, 2019). More recently a different grouping of these patient was proposed, based on age and ovarian sensitivity to OS, two features that may impact the prognosis (Esteves et al., 2019).

Surely POR still represent one of the most difficult subgroups of IVF patients to treat in the everyday clinical practice. Therefore, we set up this Research Topic with the aim to provide a comprehensive overview of the diagnostic and therapeutic management of patients with DOR and POR from different perspectives: definition, diagnostic and etiology of DOR, efficacy of different ART for the patient's management; and lastly, novel and promising strategies for the treatment of DOR and POR.

As in many other situations, in IVF the capacity to predict a possible failure is crucial. With the objective to prevent a critical outcome, the first step is to define DOR and describe which test can be performed to diagnose women with DOR. Moreover, in the literature, the clinical use of ovarian reserve markers (ORMs) is based on the use of cut-off points. However, the cut-offs are very frequently arbitrary, depending on the different definitions of DOR, the different measuring methods

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and lastly the high heterogeneity of the population investigated. For the definition and diagnosis of DOR, Wang et al., explored the ovarian reserve tests (ORTs) and their respective values, also according to specific age cut-offs, in order to predict poor ovarian response and to personalize IVF treatment appropriately. The main result showed that age, AFC, AMH and basal FSH are predicting factors for POR, where AFC and AMH are the best, if using only a single factor as predictor. AMH has a very low intra- and inter-cycle variability, thereby offering a good quantitative and qualitative follicle marker compared to clinical and endocrine ones; it is therefore the best single predictor of POR. Along similar lines of predicting ovarian response, Wen et al., investigated the reference range and the potential value of inhibin B, a non-steroidal hormone produced by the granulosa cells with a known property of FSH suppression. The main results showed that a reduction in inhibin B reflects DOR and has a good consistency with both AMH and AFC. Bai et al. , explored the ovarian response-related risk factors. They determined the expression of growth differentiation factor-8 (GDF-8), a member of the transforming growth factor β family and known to have a crucial role in folliculogenesis, and the expression of its specific receptors in different ovarian response patients during OS. The authors concluded that aging, obesity, endometriosis, ovarian surgery, and high levels of GDF-8 are high risk factors for POR.

When looking at etiology, one of the causes of DOR is the exposure to gonadotoxic medication for oncological reasons. Chemotherapy-associated ovarian failure (COF), has been described by Mauri et al. as a disruption of ovarian function both as an endocrine gland and as a reproductive organ. The real underlying mechanism by which this happens is still not fully understood; however, it seems to be associated with either DNA damage of the premature ovarian follicle or its early activation and apoptosis, resulting in the exhaustion of the follicle reserve. As a matter of fact, due to the delay in the pregnancy wish and due to the increasing percentage of women affected by malignancies, it is of the utmost importance to give any female cancer patient the opportunity to express their pregnancy wishes after any antineoplastic treatment is completed.

The definition of a unified treatment approach for POR has not yet been outlined. Given the heterogeneity of the ovarian response in the DOR population, it is questionable whether the "one size fits all" approach should still be the main research focus, or whether more refined and personalized treatment strategies should be investigated. In this direction, Papageorgiou et al. pointed out that proper molecular testing should be performed. Regulators of follicle maturation could potentially be used as prognostic biomarkers of the response to different gonadotropin regimens. In particular, the PI3K/Akt/mTOR and Hippo pathways could be monitored, as the dynamic balance between these two opposite modulators is pivotal for proper follicle maturation. However, in the absence of defined protocols based on molecular biomarkers, current research is spread over a range of heterogeneous treatment strategies.

A first line of research compared one conventional GnRH antagonist stimulation with multiple minimal OS, demonstrating the superiority of conventional OS in terms of number of oocytes retrieved and pregnancy rates Liu et al..

A second group of studies investigated the role of androgen supplementation in DOR. Despite promising results on animals, Neves et al., by reviewing the literature on DHEA, showed that there were inconclusive results on humans, due to the large heterogeneity between the studies. Notably, Chen et al., demonstrated that a faster increase in testosterone levels, from baseline to the day after the ovulation trigger, could be associated with better pregnancy outcomes.

A third research cluster aims to stimulate follicular development by triggering paracrine signaling mechanisms with either inhibition of molecular pathways together with *in vitro* activation (IVA), mechanical fragmentation, administration of bone marrow-derived stem cells (BMDSC) as well as of platelet-rich plasma (PRP) Polonio et al.; Fàbregues et al.. Although promising, such treatments are still experimental and further research is needed before translation in a clinical setting.

Finally, there are a number of stand-alone studies, possibly pioneering new frontiers in the treatment of DOR and POR. Zhu et al., found that growth hormone (GH) administration before frozen-thawed transfer would increase oocyte quantity and quality, thus improving cycle and pregnancy outcomes. Song et al., compared traditional Chinese formula Ding-Kun Pill (DKP) supplementation versus placebo in POSEIDON group 4 women and found a higher ongoing pregnancy rate in the DKP group, though the finding is based on a subgroup analysis with small sample sizes. Yang and co-workers investigated pharmacological mechanisms through which melatonin could improve ovarian reserve: in summary, melatonin was able to show anti-aging, anti-apoptotic, endocrine, and immune system regulation Yang et al. Lastly, Christodoulaki et al., proposed germline nuclear transfer (NT) as a promising new treatment for DOR patients. NT consists in the transfer of a nuclear genome from patient oocytes to enucleated donor oocytes, thus circumventing the biochemical issues related to advanced maternal age and reduced oocyte competence.

In conclusion, DOR and POR represent one of the hardest challenges in ART. As a general recommendation, a thorough exploration of the ovarian reserve and related biomarkers should always be performed as the first step towards tailored treatment strategies. However, the success rate in this population of patients is still unacceptably low. In recent years, the need for tangible improvements have pushed forward the boundaries of research and innovation. We are still at the stage of growth and exploration; however, the impressive bulk of research makes us confident that such collective effort will inevitably lead to successful outcomes in the near future.

AUTHOR CONTRIBUTIONS

All the authors substantially revised the manuscript, have approved the submitted version, and have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work. RA and PNP made substantial contributions to the evaluation and interpretation of the articles included in the Topic, and critically appraised the results in the context of scientific literature. RA wrote the editorial. PNP and SD revised the final draft of the manuscript.

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MINI REVIEW
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Chemotherapy Associated Ovarian Failure

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As the incidence of malignancies in young adults is increasing, fertility preservation in cancer survivors arises as a major concern. Especially among female cancer patients, pregnancy rates are estimated to be 40% lower compared to women of the same age. Nowadays oncologists are to be preoccupied not only with their patients' successful treatment, but also with the maintenance of the potential of the latter to conceive and obtain children. Chemotherapy associated ovarian failure (COF), refers to disruption of ovarian function both as an endocrine gland and as a reproductive organ, due to previous exposure to chemotherapy agents. Although the underlying mechanism is not fully understood, it is supposed that chemotherapy agents may induce either DNA damage of premature ovarian follicle or early activation and apoptosis of them, resulting into early exhaustion of available follicle deposit. Various chemotherapy agents have been associated with COF with the highest incidence being reported for patients undergoing combination regimens. Although a variety of alternatives in order to maintain ovarian function and fertility in female cancer survivors are available, adequately established practices to do so are lacking. Thus, it is of major importance to investigate further and collect sufficient evidence, aiming to guide patients and physicians in everyday clinical practice.

Keywords: ovarian reserve, ovarian failure, sterility, cancer, chemotherapy

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INTRODUCTION

Over 6.6 million women are estimated to be annually diagnosed with cancer, about 10% of them being younger than 40 years old (1). On the other hand, in modern Western societies, an increasing proportion of women delay their first pregnancy until the fourth decade of life (2). Notably, female cancer survivors are 40% less probable to become pregnant, compared to healthy women, with low pregnancy rates mainly reported among patients diagnosed with leukemia, cervical and breast cancer (3, 4). In this context, nowadays oncologists are not only to be preoccupied with their

patients' successful treatment, but also with major fertility preservation concerns. In the following paragraphs, we attempt to summarize the underlying mechanisms of COF, as well as the current therapeutic and preventive strategies addressing female fertility maintenance dilemmas.

COF—DEFINITION AND ASSOCIATED ANTINEOPLASTIC AGENTS

Chemotherapy associated ovarian failure (COF) refers to disruption of both endocrine and reproductive ovarian function, after exposure to chemotherapy. It is defined as either the absence of regular menses in premenopausal female patients or as increased FSH levels (>40 IU/L) (5).

In 2006, the American Association of clinical oncology attempted to sort antineoplastic regimens, according to the associated fertility compromise risk. Hematopoietic stem cell transplant (HSCT) initiation regimens steadily compromise patients' fertility, while gonadotoxicity of adjuvant chemotherapy regimens against early breast cancer varies with duration of exposure and patient's age. Characteristically, triple agent combinations, such as CMF (cyclophosphamide, methotrexate, fluorouracil), entail a high risk of infertility if administered for more than four cycles in women older than 40, whereas the risk is significantly reduced for younger patients. Notably, vincristine, methotrexate, and fluorouracil do not impose considerable fertility hazards, while there are no sufficient date regarding taxanes, oxaliplatin, and targeted treatments (6) (**Table 1**).

Considering the finite number of follicles available in the ovaries and their co-existence in different stages of development, variable pathophysiologic mechanisms have been proposed to underlie chemotherapy induced ovarian failure (see **Table 2**). These include:

a. "Accelerated" ovarian follicle maturation: Chemotherapy agents induce apoptosis of mature, functioning ovarian follicles, resulting in depression of estrogen and anti-müllerian hormone negative feedback on the gonadotropic cells of the anterior pituitary. Constantly elevated gonadotropins may accelerate maturation of premature ovarian follicles, which, in their turn, enter apoptosis under systematic chemotherapy, thus the gradual exhaustion of ovarian follicles deposit (5, 7, 8). Supporting evidence comes from histology studies of murine ovarian tissue, in cyclophosphamide treated mice, showing increased population of early growing follicles, in parallel with elimination of the quiescent ones (8). The enhanced phosphorylation of proteins involved in the maturation of primordial follicles seems to be mediated *via* the PI3K/PTEN/Akt signaling pathway, which may also be activated due to a direct effect of chemotherapy on oocytes and on pregranulosa cells supporting them (7–9).
b. Direct quiescent follicle DNA damage: Non-cell cycle specific chemotherapeutics, such as alkylating agents and doxorubicin, can induce formation of cross-links in the DNA of non-dividing, dormant oocytes. The subsequent accumulation of DNA strand breaks activates the pro-apoptotic intracellular pathways, leading to apoptosis of the affected ovarian follicles (10). Relevant supporting evidence derives from studies of human oocyte *in vitro* cultures and human ovarian xenograft murine models, exposed to doxorubicin (11) and cyclophosphamide (12), revealing double strand breaks and features of apoptotic death in premature oocytes.

TABLE 1 | Risk of infertility associated with antineoplastic systematic treatment [based on American Society of Clinical Oncology Recommendations on Fertility Preservation in Cancer Patients, (6).

Risk category	Related malignancies	Chemotherapy regimens	Patients age
High risk >80%	Various hematologic malignancies or solid tumours	HSCT initiation including cyclophosphamide/total body irradiation or cyclophosphamide/busulfan	NR
	Adjuvant early breast cancer chemotherapy	CMF, CEF, CAF ×6 or more cycles	40 yrs or older
Intermediate risk 20–80%	Adjuvant early breast cancer chemotherapy	CMF, CEF, CAF ×6 or more cycles	30 to 39 yrs
		AC ×4 cycles	40 yrs or older
Low risk <20%	Non-Hodgkin lymphoma	CHOP ×4–6 cycles, CVP	NR
	Hodgkin lymphoma	ABVD ×4–6 cycles	
	Acute myeloid leukemia	Anthracycline and cytarabine	
	Acute lymphocytic leukemia	Multi-agent	
	Adjuvant early breast cancer chemotherapy	CMF, CEF, CAF ×6	30 yrs or younger
		AC ×4	40 yrs or younger
Very low or low risk	Germ cell tumors, GI tumors	Vincristine, Methotrexate, Fluorouracil	–
Unknown	GI tumors, breast cancer, lung cancer	Taxanes	–
		Oxaliplatin	
		Irinotecan	
	GI tumors, breast cancer, melanoma, lung cancer	Monoclonal antibodies (trastuzumab, bevacizumab, cetuximab)	–
		Tyrosine kinase inhibitors	

HSCT, hematopoietic stem cell transplant; NR, not reported; CMF, cyclophosphamide, methotrexate, fluorouracil; CEF, cyclophosphamide, epirubicin, fluorouracil; CAF, cyclophosphamide, doxorubicin, fluorouracil; AC, doxorubicin, cyclophosphamide; ABVD, doxorubicin, bleomycin, vinblastine, dacarbazine; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; CVP, cyclophosphamide, vincristine, prednisone.

TABLE 2 | Summary of the suggested underlying mechanisms by which chemotherapy compromises follicular ovarian reserve (5, 7–13).

Proposed mechanism	Outline	Reference
"Accelerated" ovarian follicle maturation	Chemotherapy→ apoptosis of functioning ovarian follicles→ ↓estrogen, anti-Müllerian hormone→ ↑ gonadotropins → accelerated maturation of premature ovarian follicles→ apoptosis → gradual exhaustion of ovarian follicles deposit	Cui et al. (5) Roness et al. (7) Kalich-Philosoph et al. (8)
	Chemotherapy → ↑ activation of the PI3K/PTEN/Akt signaling pathway in oocytes and pregranulosa cells → phosphorylation of maturation mediators → apoptosis of mature follicles → gradual exhaustion of ovarian follicles deposit	Roness et al. (7) Kalich-Philosoph et al. (8) Adhikari et al. (9)
Direct quiescent follicle DNA damage	Alkylating agents, doxorubicin→ formation of DNA cross-links in non-dividing, dormant oocytes→ accumulation of DNA strand breaks→ pro-apoptotic intracellular pathways activation→direct apoptosis of quiescent follicles→ gradual exhaustion of ovarian follicles deposit	Bedoschi et al. (10) Soleimani et al. (11) Titus et al. (12)
Disrupted ovarian vascularization	Chemotherapy→ ovarian vascular spasm → ovarian ischemia related damage	Bedoschi et al. (10) Bar-Joseph et al. (13)
	Chemotherapy→fibrosis of the ovarian cortex→ compromised blood vessel formation→ ovarian ischemia related damage	
	Chemotherapy→ inhibition of angiogenesis → reduced ovarian blood flow→ ovarian ischemia related damage	

c. Disrupted ovarian vascularization: Chemotherapy may compromise the functionality of ovarian vasculature and stroma supporting the gonadal cells. Local vascular spasm reducing ovarian blood flow, fibrosis of the ovarian cortex affecting blood vessel formation, inhibition of angiogenesis, are some of the described associated mechanisms. Relative evidence has been found in *in vitro* and murine xenograft studies of human ovarian tissue, as well as mouse ovaries, exposed to doxorubicin (10, 13).

OVARIAN FUNCTION PRESERVATION APPROACHES

GnRH Analogs: Attempting to Block Premature Follicle Activation

Constant GnRH analogs administration during chemotherapy has been thought to inhibit early ovarian follicle recruitment, by desensitizing hypophysis to the innate GnRH effect (7). GnRH analogs have been mostly employed for fertility preservation in early breast cancer and lymphoma female patients, with ambiguous results.

About 20.038 women aged between 15 and 44 years are yearly diagnosed with early breast cancer in the US, and 97% of them face a risk of infertility due to adjuvant chemotherapy, while half of them wish to have children (14). The potential protective effect of GnRHa administration concurrently with adjuvant chemotherapy, in order to protect ovarian function, has been addressed in several clinical trials (15–17).

A metanalysis of seven placebo-controlled, randomized clinical trials, recruiting 1,047 patients, conducted between 1975 and 2015 seems to favor GnRH administration (15). GnRH analogs employed included goserelin (three trials), triptorelin (three trials), and leuprolide (one trial), while chemotherapy consisted mainly of anthracyclines, cyclophosphamide, and taxanes. Use of tamoxifen was reported in six trials (0% in three trials and about 70% in another three trials). GnRHa administration seemed to double rates of regular menstruation, compared to placebo, at 6 (OR = 2.41, 95% CI 1.40–4.15, p = 0.002) and 12 months (OR = 1.85, 95% CI 1.33–2.59, p = 0.0003) after chemotherapy withdrawal. Patients on GnRHa during adjuvant chemotherapy also seemed to have almost twice the chance of pregnancy, compared to the untreated women (OR 1.85, 95% IC 1.02–3.36, p = 0.04) (15, 17).

In a more recent report of the PROMISE-GIM6 trial, included in the above metanalysis, neither recovery of menstruation (HR of 1.28, 95% CI 0.98–1.68, p = 0.071) nor pregnancy rates (2.56, 95% CI 0.68–9.6, p = 0.142) were found significantly higher in triptorelin treated patients, at 7 years of follow-up (18). Nonetheless, patients' age may act as a confounding factor; in the OPTION trial (19), goserelin administration conferred an advantage in patients younger than 40 years old, as COF was observed at 2.6% of goserelin treated patients and at 20% (p = 0.038) of placebo treated patients, while no benefit was established in the total trial population (COF incidence in 18.5 *vs.* 34.8% in the goserelin and control arm, respectively, p = 0.048).

Hodgkin and non-Hodgkin lymphomas are estimated to affect up to 18 women aged between 15 and 39 years old, per 100.000 of population (20, 21). A metanalysis of three randomized clinical trials and four case-control series, including a total of 434 lymphoma patients under systematic chemotherapy, deduced that GnRHa treatment seemed to decrease the incidence of COF, defined as increased FSH level, by 68% (OR 0.32 95% CI, 0.13–0.77, p = 0.01) (22). In contrast, spontaneous pregnancy rates were not significantly affected with 13.5 and 11% of survivors getting pregnant in GnRHa and placebo treated groups, respectively (OR = 1.11, 95% CI 0.55–2.26, p = 0.75). Nevertheless, the most recent, randomized, multicenter clinical trial addressing COF in female patients having undergone chemotherapy for Hodgkin or non-Hodgkin lymphomas suggests otherwise (23). Among 67 evaluable lymphoma female patients treated with alkylating agents between 2002 and 2008, COF (defined as at least one measurement of FSH level >40 IU/L) occurred in 19.5 and 25% of patients in the triptorelin and control arm respectively. Triptorelin administration was not an independent prognostic factor for patient protection from COF, in the multivariate analysis (OR = 0.7, 95% CI 0.15–3.24, p = 0.651); in this trial, occurrence of COF after chemotherapy was found to be increased 70-fold after an initiation regimen for hematopoietic stem cell transplant, and 10-fold by administration of a

cumulative dose of cyclophosphamide greater than 5 g/m2. In addition, both groups achieved similar pregnancy rates (53% in the triptorelin treated patients, and 43% in the placebo, p = 0.467), three pregnancies occurring among placebo treated women diagnosed with protocol defined COF.

There is only one small, randomized clinical trial addressing the effectiveness of GnRHa in patients treated for ovarian cancer, with conservative surgery and adjuvant chemotherapy. Thirty patients aged between 12 and 45 years, among whom 20 were diagnosed with germ cell tumors, were 1:1 randomized to receive the GnRHa diphereline or nothing, during chemotherapy. Employed regimens included BEP (bleomycin, etoposide, cisplatin) (13 in GnRHa arm/9 in control arm), Carboplatin plus paclitaxel (2 in GnRHa arm/4 in control arm), cisplatin plus paclitaxel (0 in GnRHa arm/1 in control arm), VAC (0 in GnRHa arm/1 in control arm). COF was defined as permanent absence of menses and FSH higher than 20 mIU/ml at 6 months after chemotherapy completion. All patients receiving diphereline experienced recovery of menses, and had premenopausal FSH and estradiol values, whereas one third of patients in the control group had permanent cessation of menses, high FSH, and low estradiol levels. Remarkably, cyclophosphamide and cisplatin, the two most gonadotoxic agents, were administered only in two patients, both of them in the control group, while it is not reported if these two patients were among the five ones experiencing permanent COF (24).

Moreover, in a small study pre- and post-menarchal patients treated for Hodgkin and non-Hodgkin lymphoma, thymoma, acute myeloid, and lymphoid leukemia, GnRH treatment confined a more notable benefit in preserving menstruation and fertility in postpubertal patients, whereas prepubertal girls seemed to be at less risk of COF, even in the absence of GnRH treatment (25).

In conclusion, GnRH analog treatment is not adequately established and it is not currently suggested as a reliable measure of fertility preservation by international guidelines, although it appears to have some protective effect, especially in younger patients. More studies and more long-term results of the already conducted trials are needed to further investigate this question.

Oocyte/Embryo Cryopreservation

Oocyte or embryo cryopreservation may be recommended to premenopausal women affected by any type of malignancy (4). Oocyte cryopreservation is performed by ovarian hyperstimulation by gonadotropins and freezing of the transvaginally retrieved mature oocytes. The embryo cryopreservation protocols include *in vitro* insemination of the collected oocytes before storage. When conception is desired, either defrosted *in vitro* fertilized oocytes or defrosted embryos are introduced in the patient (26). Little is known about the potential of the ovarian stimulation to promote growth of hormone-driven neoplasms, suggesting that this strategy should probably be withheld for aggressive and hormone sensitive disease (4, 27).

The oocyte cryopreservation protocol (26) begins with controlled ovarian stimulation of the patient, by administration of FSH, follitropin alpha, lutropin alfa, and urofolitropin, starting at 2–3 days after the onset of menstruation. Mature oocytes then transvaginally collected under ultrasound guidance, after hCG administration. Oocyte insemination for embryo preservation is achieved *via in vitro* intracytoplasmic sperm injection (ICSI). Oocytes or embryos are then exposed to an ethyl glycol and dimethylsulphoxide solution and inserted in storage straws, within which they are frozen by immersion in liquid nitrogen.

Frozen eggs and embryos can be rewarmed by insertion in culture dishes, within sucrose-based culture media. Next, *in vitro* fertilized oocytes or embryos are re-introduced in the patient, after sufficient preparation with systematic and transvaginal estradiol administration.

Encouragingly, frozen oocytes are equally prone to *in vitro* fertilization compared to fresh ones (70 *vs* 72%), and even more fruitful considering embryo implantation rates (43 *vs* 35%) as well as clinical pregnancies achieved per transfer (57 *vs* 44%) (28). Besides, among 900 children born by 2009, employing cryopreservation methods, congenital anomalies rate did not differ significantly from the general population (29). However, the effectiveness of cryopreservation among female cancer survivors has not been systematically recorded (30). In a retrospective trial, performed in a tertiary care referral center, only 11 of 252 premenopausal female cancer patients attempted fertilization after cancer remission, four of them achieving pregnancies, and two ending up with a healthy delivery (31). Accordingly, oncologic female patients tend to accomplish lower implantation rates (32.5 *vs* 42.6%) as well as fewer pregnancies (35.7 *vs* 57.7%) and live deliveries (41.1 *vs* 68.8%) compared to age matched controls. In spite of these limitations, oocyte/embryo cryopreservation in cancer patients should be encouraged, as they may offer the patient a fair chance of preserving their fertility (32).

Cryopreservation of Ovarian Tissue

Cryopreservation of ovarian tissue, aspires to fully recover the ovarian endocrine and reproductive function, after being re-transplanted to the patient. Markedly, it is applicable to prepubescent girls, while not requiring potentially harmful hormonal pretreatment (33).

Indeed, 130 live births have been described worldwide, resulting from transplantation of cryopreserved ovarian tissue (33). Normal ovarian function is restored in 64% of patients undergoing autotransplantation, 58% of them achieving uncomplicated childbearing and delivery (34).

The procedure consists of laparoscopic ovariectomy, followed by dissection and vitrification of the obtained ovarian tissue (35). When restoration of the ovarian reproductive function is desired, vitrificated ovarian tissue is warmed, inoculated *in vitro* with Akt stimulators, and laparoscopically inserted in the subserosa of the fallopian tubes. After ultrasonographic confirmation of follicle maturation, the latter are transvaginally collected, *in vitro* fertilized, and re-introduced to the patient (35). Unfortunately, there are no valid biomarkers to assess the residual follicles deposit in the preserved tissue, in order to predict the expected patient's potential to produce mature follicles (33, 35).

A key question about cryopreservation is the establishment of an optimal freezing protocol, as too slow and too rapid freezing

procedures may cause osmotic cell dehydration and intracellular water crystal formation, respectively, both being detrimental to the ovarian tissue. Thus, most protocols include the use of cryoprotectants, such as glycerol, DMSO, and ethylene glycol, although at high concentrations such substances also exert a toxic effect on the ovarian tissue, creating another concern for clinical practice (33).

Vitrification is an alternative cryopreservation method consisting in the conversion of the resected ovarian tissue to a preservable glass-like solid, by ultrafast cooling in the presence of high levels of cryoprotectants (33). Despite appearing as a promising choice it has not been adequately evaluated in clinical practice. In a series of 37 patients undergoing vitrification for primary ovarian insufficiency (POI), published in 2015, IVF and embryo transfer were finally performed in four of them, resulting in three pregnancies, two of which leading to live births and one ending up with a miscarriage (35). In an earlier series of 27 POI patients, one live delivery was noted, among three patients undergoing IVF and embryo transfer (36).

In conclusion, cryopreservation of ovarian tissue is an alternative solution for fertility preservation, applicable to prepubertal patients, which should be further investigated, in order to overcome technical obstacles and obtain relevant clinical experience (33, 35).

ALTERNATIVE THERAPEUTIC APPROACHES—PRECLINICAL DATA

Except from the GnRH analogs, other pharmaceutical agents have been explored in the preclinical setting within the last 20 years, in the context of fertility preservation (see **Table 3**). These include:

Sphingosine-1-phosphate (S1P): The sphingomyelinase pathway may mediate the activation of cell death in primordial follicles, *via* accumulation of ceramide, an apoptotic molecular messenger, produced by sphingomyelinase catalyzed hydrolysis of the cellular membrane. Indeed, murine oocytes in which the sphingomyelinase gene has been either knocked down or inhibited by the molecule S1P resisted normal developmental apoptosis during gametogenesis. Similarly, in murine models treated with S1P, primordial ovarian follicles also resisted radiation induced apoptosis. Consequently, S1P may be a promising agent to be further investigated in future studies, although its anti-apoptotic effect may potentially compromise the cytotoxicity of chemotherapy agents (37).

Imatinib: a widely used tyrosine kinase inhibitor, has been thought to exert an anti-apoptotic effect in primordial ovarian follicles, through inhibition of c-ABl kinase mediated apoptotic pathway. Imatinib co-administration with cisplatin to rodent models can limit death of primordial follicles, preserving reproductive ovarian function (38), although these results were not replicated (39).

AS101: AS101 acts as a modulator of the PI3K/PTEN/Akt pathway, mediating primordial follicle activation under chemotherapy. Supportively, when administered to female rodents under cyclophosphamide treatment, AS101 was found to reduce activation and subsequent exhaustion of ovarian quiescent follicles, thus preserving fertility (20) without compromising the effectiveness of antineoplastic treatment (40).

G-CSF: Interestingly, Granulocyte colony stimulating factors, frequently used against chemotherapy induced myelotoxicity, can maintain ovarian function in mice models under treatment alkylating factors, by promotion of neovascularization of the ovarian tissue (41), what may protect the ovaries from chemotherapy related ischemia.

Tamoxifen: Tamoxifen, an estrogen antagonist used in hormone-dependent breast cancer, has been also explored as a potential fertility preservation agent. As it has been shown in rodent studies, co-administration of tamoxifen with cyclophosphamide and doxorubicin seems to preserve ovarian follicle deposit (42). Although the underlying mechanism has not been clarified, it has been suggested that tamoxifen upregulates

TABLE 3 | Summary of the alternative therapeutic agents under preclinical investigation (7, 20, 37–42).

Investigated alternative agent	Mechanism of action	Potential drawbacks	Reference
S1P	Inhibition of sphingomyelinase → reduced hydrolysis of the cell membrane lipids→reduction of the pro-apoptotic molecule ceramide→ limitation of primordial follicles cell death	S1P anti-apoptotic effect may antagonize the cytotoxicity of chemotherapy agents	Morita et al. (37)
Imatinib	Inhibition of c-ABl kinase → apoptotic pathway blockade in primordial follicles	Results not replicated in more recent experiments	Gonfloni et al. (38) Kerr et al. (39)
AS101	Reduced activation of the PI3K/PTEN/Akt pathway→reduced primordial follicle maturation →reduced accelerated maturation and death of quiescent follicles	Not reported—actually it may exert an anti-tumor effect	Eichenauer et al. (20) Carmely et al. (40)
G-CSF	↑neovascularization of the ovarian tissue→ protection from ischemia	Not reported	Skaznik-Wikiel et al. (41)
Tamoxifen	Estrogen antagonist- potentially up-regulates IGF-1→ protection of primordial follicles from oxidative stress	Not reported	Roness et al. (7) Ting et al. (42)

S1P, sphingosine-1-phosphate; AS101, ammonium trichloro(dioxoethylene-o,o')tellurate; G-CSF, Granulocyte colony-stimulating factor; IGF-1, Insulin-like Growth Factor 1.

IGF-1 (Insulin-like Growth Factor 1), which protects primordial follicles from oxidative stress (7).

CONCLUSION—FURTHER QUESTIONS

Although a variety of alternatives in order to maintain ovarian function and fertility in female cancer survivors, diagnosed and undergoing chemotherapy at a young age, adequately established practices to do so are lacking. Notably, study of the applicable literature reveals a relative lack of clinical evidence regarding preservation of patient fertility among a variety of malignancies mostly affecting children, adolescents, and young adults of both genders, such as CNS tumors, germ cell neoplasms, osseous and soft tissue sarcomas. Similarly, fertility preservation in young patients affected by cancer types more frequent in older ages, such as early stage colon cancer, has not been investigated sufficiently either.

Although current oncofertility guidelines are universal among different tumor types and patient profiles (43), potential disparities between patients due to age, chemotherapy agents employed, and the malignancy itself may also interfere with fertility preservation practices. Consequently, a more methodical investigation of fertility preservation strategies, considering the above parameters, is required, in order to adequately establish the most efficient practices for each patient group.

Especially regarding young female cancer survivors, in an era that age of pregnancy is pushed even after the age of 40, it is of major importance to further investigate and collect sufficient evidence, aiming to safely guide patients and physicians in everyday clinical practice. Until then, oncologists should not neglect this domain of life of their female, younger patients; female cancer patients have to be encouraged to express their concerns and wishes, regarding fertility and pregnancy after antineoplastic treatment completion, in order to organize a plan of action that will allow them to maintain a normal endocrine function as well as the possibility to create a family.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Cumulative Live Birth Rate and Cost-Effectiveness Analysis of Gonadotropin Releasing Hormone-Antagonist Protocol and Multiple Minimal Ovarian Stimulation in Poor Responders

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Background: The overall cumulative live birth rate (CLBR) of poor ovarian responders (POR) is extremely low. Minimal ovarian stimulation (MOS) provides a relatively realistic solution for ovarian stimulation in POR. Our study aimed to investigate whether multiple MOS strategies resulted in higher CLBR compared to conventional gonadotropin releasing hormone (GnRH) antagonists in POR.

Methods: This retrospective study included 699 patients (1,058 cycles) from one center, who fulfilled the Bologna criteria between 2010 and 2018. Overall, 325 women (325 cycles) were treated with one-time conventional GnRH antagonist ovarian stimulation (GnRH-antagonist). Another 374 patients (733 cycles) were treated with multiple MOS including natural cycles. CLBR and time-and-cost-benefit analyses were compared between these two groups of women.

Results: GnRH antagonists provided more retrieved oocytes, meiosis II oocytes, fertilized oocytes, and more viable embryos compared to both the first MOS ($p < 0.001$) and the cumulative corresponding numbers in multiple MOSs ($p < 0.001$). For the first *in vitro* fertilization (IVF) cycle, GnRH antagonists resulted in higher CLBR than MOS [12.92 *versus* 4.54%, adjusted OR (odds ratio) 2.606; 95% CI (confidence interval) 1.386, 4.899, $p = 0.003$]. The one-time GnRH-antagonist induced comparable CLBR (12.92 *versus* 7.92%, adjusted OR 1.702; 95% CI 0.971, 2.982, $p = 0.063$), but a shorter time to live birth [9 (8, 10.75) months *versus* 11 (9, 14) months, $p = 0.014$] and similar financial expenditure compared to repeated MOS [20,838 (17,953, 23,422) ¥ *versus* 21,261.5 (15,892.5, 35,140.25) ¥, $p = 0.13$].

Conclusion: Both minimal ovarian stimulation (MOS) and GnRH-antagonists provide low chances of live birth in poor responders. The GnRH antagonist protocol is considered a

suitable choice for PORs with comparable CLBR, shorter times to live birth, and similar financial expenditure compared to repeated MOS.

Keywords: *in vitro* fertilization, poor ovarian responders, gonadotropin releasing hormone-antagonist, minimal ovarian stimulation, cumulative live birth rate

INTRODUCTION

Approximately 20% of all women undergoing assisted reproductive technology (ART) treatment demonstrate a poor ovarian response with very few retrieved oocytes, which are of low-quality. Most of these patients have poor ovarian reserve (1). In studies, some poor responders were retrospectively identified after some form of conventional ovarian stimulation. Patients with advanced age or abnormal ovarian reserve tests are more appropriately defined as expected poor responders. The Bologna criteria have been validated to represent a homogenous population with a uniform poor prognosis and similar clinical outcomes. According to the Bologna criteria of the European Society of Human Reproduction and Embryology (ESHRE) consensus, "poor response" to ovarian stimulation for *in vitro* fertilization (IVF) is defined by the presence of at least two of the three following features: 1) age ≥ 40 years or any other risk factor for POR, 2) ≤3 oocytes retrieved previously after conventional stimulation, and 3) antral follicle count (AFC) < 5–7 follicles or anti Mullerian hormone (AMH) < 0.5ng/ml (2).

These patients represent a conundrum in modern IVF. Studies on ART did not provide solid evidence for the preferred strategy and definite solutions for parenthood in these patients, considering the limited supply of oocytes, poor quality of embryos, and high frequency of canceled cycles. However, adjuvant treatments such as growth hormone (GH), dihydroepiandrosterone (DHEA), and CoQ10 have been claimed to be co-treatments of choice for controlled ovarian stimulation (COS) in these patients, and have shown somewhat better clinical results in some studies in terms of achieving pregnancy (3–5). However, the overall pregnancy rate per cycle in PORs is still extremely low, and varies from 7.6 to 17.5% compared to 25.9–36.7% in normal responders (6). The drop-out rate in this population of women is as high as 25% worldwide. In practice, the low live birth rate varies between different POSEIDON groups; this is mainly attributed to maternal age and ovarian response. It is of utmost importance to provide effective and patient-friendly alternative treatment options for poor responders based on the couple's genetic material.

Several ovarian stimulation protocols have been investigated, including either gonadotropin-releasing hormone (GnRH) agonists or antagonists; however, no consistent results have been reported (7–10). Recently, the DuoStim strategy, which involves luteal-phase stimulation (LPS) and follicular-phase stimulation (FPS) in one single cycle, has been reported to be promising in that it avoids discontinuation after failed attempts and slightly increases the cumulative live birth rate (CLBR) per intention to treat (11). However, cost-benefit analysis and more randomized controlled trials are needed to verify the effectiveness and safety issues. Previous data have demonstrated that increased starting doses in predicted poor responders to IVF/intracytoplasmic sperm injection (ICSI) did not increase the live birth rate, but was more highly priced (12, 13). Although studies on minimal ovarian stimulation (MOS) or modified nature cycles in POR are limited, they have suggested that MOS is a relatively realistic solution for parenthood in POR compared to conventional high dose stimulation. MOS showed a relatively higher implantation rate, acceptable live birth rate, and preferred cost-effectiveness, although fewer oocytes were retrieved (14–20). However, no study has evaluated the CLBR per person for multiple modified nature cycles. CLBR has been a better indicator of quality and success of IVF overall, as multiple cycles of MOS are usually performed instead of one-time stimulation; in addition, cryopreservation has become an integral aspect of IVF (21). It remains unclear whether poor responders could actually benefit from MOS. No data comparing the CLBR between multiple MOS and high-dose GnRH antagonist protocols in POR are available, and studies comparing time and cost effectiveness analysis are lacking. The aim of this study was to evaluate the CLBR and time-and-cost-benefit difference between GnRH-antagonists and multiple MOS protocols in poor responders who fulfilled the Bologna criteria. This study will help clinicians personalize and select a relatively superior COS strategy for these difficult patients.

MATERIALS AND METHODS

Participants

This retrospective study analyzed 325 poor responders who underwent 325 GnRH-antagonist cycles, and 374 poor responders who underwent 733 minimal ovarian stimulation cycles between January 2010 and June 2018 in one assisted reproduction center. Patient inclusion criteria were patients who fulfilled the Bologna criteria for the definition of POR which is defined by the presence of at least two of the three following features: 1) age ≥ 40 years or any other risk factor for POR, 2) ≤3 oocytes retrieved previously after conventional stimulation, and 3) antral follicle count (AFC) < 5–7 follicles or anti-Mullerian hormone (AMH) < 0.5ng/ml. The AFC was determined by counting follicle sized between 2 and 10 mm

Abbreviations: CLBR, cumulative live birth rate; FET, frozen-thawed transfer; MOS, minimal ovarian stimulation; GnRH-antagonist, gonadotropin releasing hormone antagonist; ART, assisted reproductive technology; COS, controlled ovarian stimulation; POR, poor ovary responder; ESHRE, European Society of Human Reproduction and Embryology; GH, growth factor; DHEA, dehydroepiandrosterone; LPS, luteal-phase stimulation; FPS, follicular-phase stimulation; ITT, intention to treat; IVF, *in vitro* fertilization; 95% CI, 95% confidence interval; PGS, preimplantational genetic screening; FET, frozen-thawed embryo transfer.

according to criteria proposed by Broekmans et al. in 2010 (22). AFC observers were trained by arranging workshop and instructions for the procedure. AMH was not included in our analysis due to the inconsistency of detection method in the hospital. Among the patients who fulfilled the Bologna criteria, our analysis included two groups of POR. The first group is poor responders in whom the first stimulation cycle was administered with the GnRH-antagonist protocol. Notably, only the first stimulation cycle namely the GnRH-antagonist cycle and the consecutive frozen-thawed embryo transfer (FET) cycles were exclusively included for this group of patients. The other group of poor responders included patients, in whom ovarian stimulation cycles exclusively involved MOS or natural cycles. Poor responders who had undergone other protocols were excluded (**Figure 1**). Additionally, patients with endometrial polyps, submucosal myomas, endometrium separation, history of multiple induced abortions (≥4 times), diagnosis of uterine adhesions, uterine malformation like Mullerian anomalies, bicornuate uterus, complete septate uterus were excluded. Patients who underwent PGT-A were also excluded. All poor responders were informed that the clinical pregnancy rate was frustratingly low, and the choices of GnRH-antagonist protocols or multiple MOS were discussed with them.

Gonadotropin Releasing Hormone Antagonist and Minimal Ovarian Stimulation Protocols

In the flexible GnRH antagonist protocol, at least 300 IU/day recombinant follicle stimulating hormone (FSH) and/or human menopausal gonadotropin were initiated on day 2 or 3 of the menstrual period and continued daily afterward until the day of human chorionic gonadotropin (hCG) administration. The dose was adjusted according to the ovarian response. Cetrorelix (0.25 mg) was started flexibly when the follicle reached a mean diameter of 14 mm, and continued daily afterward until the day of hCG administration; hCG 6,000–10,000 IU or GnRH-agonists 0.1–0.2 mg were selectively administered for final oocyte maturation when at least two follicles reached a diameter of 17 mm.

In MOS, clomiphene at a dose of 25–100 mg was started on day 2 or 3 of the menstrual period and continued daily for 5 days, or until trigger day. Gonadotropin at a dose of 75–150 IU was selectively initiated from day 3 or 5 of the menstrual period; hCG (6,000–10,000 IU) or GnRH-agonists (0.1–0.2 mg) were selectively used as a trigger for final oocyte maturation when 1–2 follicles reached a diameter of 17 mm. In natural cycles, there is no gonadotropin or clomiphene or Letrozole administered. hCG 6000 or GnRH-agonists 0.1mg were selectively administered for final oocyte maturation. Mono-follicular development was advocated for oocyte retrieval.

Luteal phase supplementation depended on fresh embryo transfer cycle or FET cycle including artificial and natural cycle. For fresh embryo transfer cycle and FET with natural cycle, luteal phase supplementation was initiated days before embryo transfer, specifically 40 mg oral dydrogesterone per day until 12 weeks of gestation and hCG 2000 IU intramuscularly every 5 days for three times. For FET-HRT cycles, once the timing of FET was determined, administration of progesterone intramuscularly 60 mg or Crinone vaginally 90 mg was initiated daily along with 40 mg oral dydrogesterone per day and 6 mg oral estradiol per day.

Oocyte Retrieval Laboratory Procedures

Oocyte retrieval was performed under ultrasound guidance 35–36 h after the trigger. IVF or ICSI was selectively used for fertilization. Embryos were either freshly transferred after oocyte retrieval or frozen-thawed transfer in consecutive FET cycles. All embryos were cultured in in 37°C, 5% O_2 and 6% CO_2 concentration. Embryo development was evaluated according to the morphological criteria. Day 2 or 3 cleavage-stage embryos with at least 3 or 6 blastomeres respectively, and less than 20% fragmentation were eligible for transfer and cryopreservation. For blastocysts, fully expanded to hatched blastocysts with inner cell mass and trophectoderm B quality (from 4BC upward) were eligible. Luteal phase supplementation was applied variably

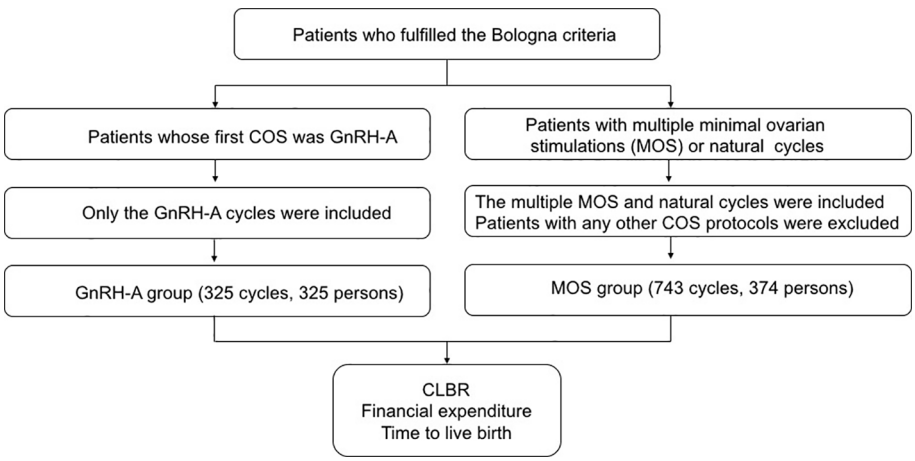

FIGURE 1 | Flow diagram of patient inclusion.

according to embryo transfer strategies and various endometrium preparation methods in FET cycles.

Outcome Measures

The primary outcome was the CLBR per aspiration for women with a GnRH-antagonist protocol, defined as at least one delivery of a live infant resulting from one ART aspiration cycle, including fresh and FET cycles within 24 months. For women administered the MOS protocol, the CLBR per person was defined as at least one delivery of a live infant resulting from all ART cycles within 24 months (21). The number of oocytes retrieved and fertilized, number of viable embryos, financial expenditure, and time to first live birth were secondary outcomes. Cycles where no oocytes were retrieved and no viable embryos were generated were also included in this study. Women who were not followed up because of loss of contact and whose frozen embryos remained un-transferred within 24 months were considered as "not having live births".

Statistical Analysis

Analyses were performed according to the intention-to-treat principle. Comparisons between GnRH-antagonists and MOS were performed using the Student's t-test, Man-Whitney U test, and chi-square tests. Student's t-test was used where sample data were normally distributed for continuous values and the mean (± SD) was reported. Man-Whitney U test was used where sample data were not normally distributed for continuous values and the median (first quartile, third quartile) was reported. Chi-square was used for categorical values and the number was reported. We verified variables distribution by statistical tests Kolmogorov-Smirnov and Shapiro-Wilk from SPSS. Univariate regression and multivariate logistic regression were applied to identify the candidate factors predictive of CLBR. The candidate variables were the age, body mass index, basal FSH, basal estradiol (E2), infertility years, primary infertility (*vs*. secondary infertility), and ovarian stimulation protocols. All independent variables were concomitantly entered into the logistic regression model. The likelihood of CLBR was presented as an odds ratio (OR) and 95% confidence intervals (CI). All analyses were conducted using SPSS statistics. P values < 0.05 were considered statistically significant. The economic analysis included costs for pharmacological compounds and IVF procedures up to the day of pregnancy. Economic evaluation focused on direct medical costs, not including the cost of examinations before IVF treatment or any commute fees. Costs were based on Shanghai General Hospital prices and have been presented in RMB.

Ethical Approval

Approval for this study was obtained from the institutional review board and ethics committee of the Shanghai General Hospital.

RESULTS

This study included 325 women (325 cycles) who underwent GnRH-antagonist ovarian stimulation and 374 patients (733 cycles) who underwent multiple minimal ovarian stimulation (MOS), including the natural cycle. Baseline demographic and clinical characteristics between the GnRH-antagonist and MOS groups were similar, although as shown in **Table 1**, the basal FSH in the MOS group was higher than that in the GnRH-antagonist group ($p < 0.001$). GnRH-antagonist cycles were characterized by significantly longer durations of gonadotropin (Gn) stimulation days, a higher total dose of Gn, higher peak E2, higher progesterone (P) levels, lower luteinizing hormone (LH) levels, and thicker endometrium than the MOS cycle at the trigger day (**Table 2**). GnRH-antagonists resulted in higher number of oocytes retrieved, meiosis II oocytes, fertilized oocytes, and more viable embryos than both the first MOS and cumulative stimulation of multiple MOSs ($p < 0.001$) (**Table 2**).

As for clinical results (**Table 3**), the CLBR for both groups of patients was low. For the first IVF cycle, GnRH-antagonists demonstrated higher CLBR per aspiration than the first MOS on both, univariate analysis (12.92 *versus* 4.54%, crude OR 3.117; 95% CI 1.737, 5.592, $p < 0.001$) and multivariate analysis after adjusting for female age, body mass index, basal FSH, basal E2, infertility years, and primary infertility (*vs*. secondary infertility) (adjusted OR 2.606; 95% CI 1.386, 4.899, $p = 0.003$). Female age, basal FSH, and infertility years were independent factors negatively associated with the likelihood of CLBR per aspiration (**Supplemental Figure 1**). A cluster of multiple aspiration cycles per woman has to be considered in the MOS group. Therefore, we also measured the CLBR per person in this group of patients. The CLBR per aspiration in the GnRH-antagonist group was higher than the CLBR per person of multiple MOSs on univariate analysis (12.92 versus 7.22%, crude OR 1.907; 95% CI 1.147, 3.171, $p < 0.001$), while the type of ovarian stimulation (GnRH-antagonist *vs*. MOS) was not associated with CLBR on multivariate logistic regression after adjusting for the same factors (adjusted OR 1.702; 95% CI 0.971, 2.982, $p = 0.063$). Female age and basal FSH were the only

TABLE 1 | Baseline demographic and clinical characteristics based on different protocols.

	GnRH-antagonist stimulation	Minimal ovarian stimulation	P
Maternal age (year)	38.46 ± 4.64	38.83 ± 4.75	0.328
Body mass index	23.57 ± 2.9	23.78 ± 3.10	0.056
Primary infertility	143	154	0.451
Infertility years	5 (2, 8)	4 (2, 7)	0.134
Primary cause of infertility			
Male	117	129	0.677
Tubal	209	231	0.487
Poor ovary response	6	25	0.002
Endometriosis	14	21	0.429
Anovulatory	10	4	0.105
Unexplained	12	4	0.039
Other causes	8	15	0.252
Basal E2 level (pmol/L)	145 (95.5, 211.5) (N=315)	134 (88.59, 211.00) (N=355)	0.220
Basal FSH level (mIU/ml)	9 (7.2, 15.1) (N=315)	11.7 (8.600, 17.725) (N=354)	<0.001

TABLE 2 | Cycle characteristics according to different protocols.

	First GnRH-antagonist stimulation (325 cycles)	Minimal ovarian stimulation		P^a	P^b
		First (374 cycles)	Multiple (733 cycles)		
Duration of Gn stimulation (days)	9 (8, 10)	6 (4, 8)	/	<0.001	/
Total dose of Gn (IU)	2400 (1800, 2925)	900 (600.00, 1256.25)	/	<0.001	/
Peak E2 level at trigger day (pmol/L)	7585 (4213.5, 11666.0) (N=320)	2707 (1630, 4815) (N=365)	/	<0.001	/
P level at trigger day (nmol/L)	2.64 (1.623, 3.683) (N=68)	1.36 (1.032, 3.105) (N=96)	/	0.005	/
LH level at trigger day (U/L)	3.14 (2.205, 5.210) (N=67)	7.96 (5.318, 13.858) (N=96)	/	<0.001	/
Endometrial thickness at trigger day (mm)	9 (8.5, 10.4)	6 (5.0, 8.2)	/	<0.001	/
ICSI/IVF	100/225	104/270	/	0.390	/
Number of oocytes retrieved	7 (4, 10)	2 (1, 4)	4 (2, 7)	<0.001	<0.001
Number of MII oocytes	5 (3, 8.25)	2 (1, 3)	3 (1, 4)	<0.001	<0.001
Number of fertilized oocytes	5 (3, 7)	2 (1, 3)	3 (1, 5)	<0.001	<0.001
Number of viable embryos	2 (1, 4)	1 (0, 2)	2 (1,3)	<0.001	<0.001

[a]*First GnRH-antagonist vs. first minimal ovarian stimulation.*
[bb]*First GnRH-antagonist vs. multiple minimal ovarian stimulation.*
Gn, gonatrodopin; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; MII, meiosis II.

TABLE 3 | Clinical outcomes according to different protocols.

	First GnRH-antagonist (325 cycles, 325 persons) (per aspiration)	First minimal ovarian stimulation (374 cycles, 374 persons) (per aspiration)	Multiple minimal ovarian stimulation (733cycles, 374 persons) (per person)	P^a	P^b	Adjusted OR (95%CI)$^aP^a$	Adjusted OR (95%CI)$^bP^b$
CLBR	42 (12.92%)	17 (4.54%)	27 (7.22%)	<0.001	0.012	2.606 (1.386, 4.899) 0.003	1.702 (0.971, 2.982) 0.063
Cost	20,838 (17,953, 23,422)	12,254 (9,612.5, 14,875.5)	21,261.5 (15,892.5, 35,140.25)	<0.001	0.130	/	/
Time to First Live Birth	9 (8, 10.75)	/	11 (9, 14)	/	0.014	/	/

[a]*First GnRH-antagonist vs. first minimal ovarian stimulation.*
[b]*First GnRH-antagonist vs. multiple minimal ovarian stimulation.*
Adjusted OR: adjusting for age, body mass index, basal FSH, basal E2, infertility years, and primary infertility (vs. secondary infertility).
CLBR, cumulative live birth rate; OR, odds ratio; CI, confidence interval.

independent factors negatively associated with the likelihood of CLBR (**Supplemental Figure 2**).

On considering the first cycle of ovarian stimulation during economic-effectiveness analysis, the cost of using GnRH antagonists was higher than that of MOS [20,838 (17,953, 23,422) ¥ *versus* 12,254 (96,12.5, 14,875.5) ¥, $p < 0.001$]. However, the cumulative financial expenditure was statistically similar between one time GnRH-antagonists and multiple MOS [20,838 (17,953, 23,422) ¥ *versus* 21,261.5 (15,892.5, 35,140.25) ¥, p=0.13]. On considering the time to first live birth, GnRH-antagonists showed obviously shorter times than repeated modified natural cycles [9 (8, 10.75) months *versus* 11(9, 14) months, $p = 0.014$].

DISCUSSION

Main Findings

In the present retrospective study on POR, patients who underwent COS with conventional GnRH-antagonist protocols had a significantly higher number of retrieved oocytes, viable embryos, and statistically similar CLBR compared to those who underwent multiple MOS; however, the time to live birth was earlier with similar financial expenditure. The GnRH-antagonist protocol is a suitable choice when developing a COS strategy plan for poor responders.

Interpretation of Data

We evaluated whether poor responders benefit from GnRH-antagonist protocols compared to MOS, as the preferred protocol in these patients remain unclear. Although reports suggest that MOS is a relatively preferable strategy for POR, we believe that controlled ovarian hyperstimulation with daily high gonadotropin doses in the GnRH-antagonist protocol should be commonly offered to poor responders. Our observations are in accordance with research that suggests that raising FSH levels during stimulation by high-dose FSH reduces cancellation and improves clinical success (23), and mild ovarian stimulation is inferior to conventional regimen in POR in terms of retrieved cumulus oocyte complexes (22, 24). In addition, there are several

studies comparing MOS and other ovarian stimulation protocols applied in POR including some RCTs (12, 15, 25–28). They suggested MOS or mild ovarian stimulation induced non-inferior successful rate with shorter duration of stimulation and economical advantages than conventional ovarian stimulation strategy (19, 29–32). The strategy of performing increasing FSH starting dose has not shown any consistent benefit. However, the main limitation of these studies is the low number of patients and the lack for data involving cryopreservation of surplus embryo and cumulative pregnancy rate. The outcomes of consecutive FET cycles are important because fresh live birth rate was negatively impacted by high dose of gonadotropin, while frozen transfer live birth rate was unaffected by total FSH dose (33). Our study is the first to evaluate the CLBR including both FET cycles and repeated MOS cycles in 2 years of follow-up.

In the context of laboratory performance, the need for the retrieval of a large number of oocytes *via* ovarian stimulation is an integral part of successful IVF treatment, since the number of oocytes and viable embryos are independent factors that increase CLBR (34). A large oocyte field is associated with an increased likelihood of CLBR per aspiration across female age. For poor responders, the pregnancy rate is reduced when fewer oocytes were retrieved. The maximum CLBR is observed when around nine oocytes are retrieved in women older than 45 years (6, 35) Any additional oocyte retrieved indicates possible improvement of CLBR for this challenging population of POR.

The higher number of euploid blastocysts correlated with a higher cumulative pregnancy rate. Reports have indicated that a higher dose of gonadotropins resulted in an increased rate of aneuploidy in embryos and granulosa cells (36). However, there are some controversies in this regard. Earlier research suggested that a higher proportion of embryos of good morphological quality are obtained with mild stimulation compared to conventional stimulation, and embryo development is adversely affected in a COS dose-dependent manner (37). However, recent studies demonstrated that aggressive stimulation does not increase the rate of embryo aneuploidy in preimplantation genetic screening (PGS) cycles in both, infertile patients and oocyte donors (38). The so-called "detrimental effect" of high dose stimulation was not evident when natural and stimulated IVF cycles were compared. The benefits of a higher number of retrieved oocytes cannot be mitigated by the age-related embryo aneuploidy rate, and can explain why high stimulation results in similar reproductive outcomes. Higher doses of gonadotropins tend to result significantly higher E2 levels on the day of hCG administration and diminished endometrial receptivity. However, the freeze-all policy and higher frequency of FET alleviates the possible negative influence of conventional high-dose stimulation on endometrial receptivity. Endometrium maybe adversely affected by high dose of gonadotropin only in fresh IVF cycle. In a retrospective analysis, Trifon et al. suggested that live births are significantly higher with modified natural cycles than with high-dose FSH stimulation GnRH-antagonists in poor responders (14). However, they only accounted for the live birth rate in fresh transfer cycles and did not consider other FET cycles, which represent the whole picture of these clinical situations.

Tilborg et al. indicated that an increased dose of FSH resulted in a statistically similar CLBR compared to the standard dose regimen, but with collateral increases in financial costs (12). Financial factors play an important role when considering the number of IVF cycles a patient will attempt, since there is no insurance coverage for IVF treatment in some countries including China. The modified natural cycle was considered to be a patient-friendly ovarian stimulation protocol. Some research has shown that multiple MOS or modified natural cycles offer a reasonable long-term success rate with less financial costs. However, a report suggested that modified natural cycles are of no benefit with a less than 1% live birth rate in genuine poor responders, who yielded up to three oocytes with conventional COS (39). The lower ongoing pregnancy rate resulting from mild stimulation was particularly related to a high cancellation rate (40). In our analysis, the total financial expenditure per person for repeated MOS was similar to that of the one-time GnRH-antagonist protocol. The drug cost linked to gonadotropin in one-time GnRH-antagonist regimen is paralleled by the clinical outcome. From our experience, in the multiple MOS strategy, the cost of repeated oocyte retrieval and embryo transfer procedures comprises most of the cumulative financial cost, while the pharmacological expenses of gonadotropin are considerably less. The whole financial expenditure of repeated MOS is not less than the conventional GnRH-antagonist regimen. Additionally, in our study, repeated MOS showed a longer time to live birth than the GnRH-antagonist protocol. Thus, repeated MOS is not as beneficial as presumed.

Strengths and Limitations

Before the IVF treatment for these poor responders, both clinicians and patients were confronted with the high possibility of repeated stimulation cycles. In the MOS group, a cluster of multiple treatment cycles per woman has to be considered. One strength of our study was that we measured the CLBR of multiple modified natural cycles, which included not only the live birth rate from one single stimulation cycle, but also that of the consecutive cycles within 2 years of follow-up. Thus our analysis contributed important data to daily clinical practice before making the ovarian stimulation strategy for these poor responders. This research is limited by its retrospective design. Patients were allocated to two stimulation protocols based on the physician's discretion and patient consultations; selection bias is therefore possible, and potential confounders cannot be accounted for. Poor responders are not a homogeneous group of patients, and the prognosis varies greatly depending on the age or actual number of oocytes obtained. Both, predicted and unexpected poor responders were included in our analysis. Unexpected poor responders seem to have different biological characteristics and prognosis as a different entity than the predicted poor responders (41). The heterogeneous population may have had different prognoses; this may have affected our inferences.

CONCLUSIONS

The current study provides evidence that GnRH-antagonists are not inferior to multiple MOS in POR in terms of both, the success rate and time-and-cost-benefit analysis. While making COS strategy plans for predicted POR, this analysis may improve the counseling of IVF treatment for these poor responders and assist clinicians in determining the best candidates for the COS strategy. The GnRH-antagonist protocol enhanced the oocytes yield, did not lead to considerable cost and acted as a reasonable alternative for this difficult-to-treat group of patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional review board and ethics committee of Shanghai General Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL and YW were involved in the study concept and design. YL collected the data and drafted the manuscript. RS analyzed the data. YW revised the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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This manuscript has been released as a pre-print at Research Square (YL, YW) (30).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.605939/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | The odds of achieving CLBR per aspiration between first GnRH antagonist administration and first MOS.

SUPPLEMENTARY FIGURE 2 | The odds of achieving CLBR per person between first GnRH antagonist administration and multiple MOS.

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Clinical Outcomes of Frozen-Thawed Embryos Generated From Growth Hormone Stimulation in Expected Poor Responders

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Objective: This study aimed to elucidate whether growth hormone (GH) adjuvant therapy significantly improves clinical outcomes for expected poor responders in frozen-thawed cycles.

Methods: Expected poor responders undergoing controlled ovarian stimulation with or without GH adjuvant therapy, and subsequently underwent the first frozen-thawed transfer from January 2017 to March 2020 were retrospectively reviewed. Maternal age was matched at a 1:1 ratio between the GH and control groups. All statistical analyses were performed with the Statistical Package for the Social Sciences software.

Results: A total of 376 frozen-thawed cycles comprised the GH and control groups at a ratio of 1:1. The number of oocytes (7.13 ± 3.93 *vs*. 5.89 ± 3.33; p = 0.001), two pronuclei zygotes (4.66 ± 2.76 *vs*. 3.99 ± 2.31; p = 0.011), and day 3 available embryos (3.86 ± 2.62 *vs*. 3.26 ± 2.04; p = 0.014) obtained in the GH group was significantly higher than the control group in corresponding fresh cycles. The clinical pregnancy (30.3 *vs*. 31.0%; p = 0.883), implantation (25.3 *vs*. 26.2%; p = 0.829), early abortion (16.1 *vs*. 15.8%; p = 0.967), and live birth rates (20.6 *vs*. 20.8%; p=0.980) were comparable between the two groups in frozen-thawed cycles. Improvement in the clinical pregnancy (46.8 *vs*. 32.1%; p = 0.075), early miscarriage (10.3 *vs*. 20.0%; p = 0.449), and live birth rates (35.7 *vs*. 18.9%; p = 0.031) was found in the subgroup of poor ovarian responders (PORs) with good quality blastocyst transfer (≥4BB) following GH co-treatment.

Conclusions: GH administration would increase oocyte quantity and quality, in turn, improve live birth rate in PORs.

Keywords: growth hormone, poor responder, frozen-thawed cycle, clinical pregnancy rate, utilization rate, live birth

INTRODUCTION

As part of *in vitro* fertilization (IVF)/intracytoplasmic sperm injection treatment, controlled ovarian stimulation (COS) was performed with exogenous follicle-stimulating hormone to obtain a sufficient number of oocytes and good quality embryos for transfer (1). Of note, there are still women who have a poor response to COS [poor ovarian responders (PORs)], resulting in only a few oocytes at the time of retrieval, a small number of embryos for transfer, a reduced pregnancy rate, and a higher treatment discontinuation rate (2–6). Thus, PORs are a significant challenge for reproductive endocrinologists and embryologists.

The feasibility of growth hormone (GH) adjuvant therapy is based on the GH requirement for follicular development and ovulation (7, 8). GH enhances the effect of gonadotrophins on follicular growth and ovulation (8). A recent systematic review and meta-analysis suggested that GH adjuvant therapy significantly increases the number of oocytes retrieved and the available embryos in PORs who fulfilled the Bologna criteria (9). Because PORs are highly heterogeneous and GH addition protocol varies from center-to-center, the efficacy of GH in improving pregnancy and live birth rate has been widely debated for a long time. Of note, previous studies from PIVET medical center, which showed a beneficial effect of GH adjuvant therapy on pregnancy and live birth rates in fresh and frozen cycles with poor prognosis patients (10, 11).

However, there is still no compelling evidence supporting the notion that improvement was due to GH action on oocyte quality. In the current study, the clinical outcomes of frozen-thawed cycles were compared, while excluding the possible effect of GH on endometrial receptiveness to elucidate whether GH adjuvant therapy significantly increased the clinical pregnancy and live birth rates by improving oocyte quality.

MATERIALS AND METHODS

Participants

Expected PORs who underwent COS with or without GH adjuvant therapy (control group), and subsequently underwent the first frozen-thawed cycle from January 2017 to March 2020, were retrospectively reviewed. Participants were included without considering pregnancy outcome in corresponding fresh cycles. Expected PORs were defined based on an anti-Mullerian hormone (AMH) < 1.2 ng/ml and an antral follicle count (AFC) < 7. Maternal age was matched at a 1:1 ratio for the GH and control group (without GH adjuvant therapy). Patients with azoospermia or severe oligospermia and patients undergoing pre-implantation genetic diagnosis were excluded. The study group consisted of 188 PORs undergoing GH adjuvant therapy and the control group consisted of 188 PORs without GH adjuvant therapy. Patients who were offered GH administration had undergone 1.86 IVF cycle attempts; patients in the control group had 1.70 IVF cycle attempts before enrollment in this study.

Clinical Protocol

COS was achieved in those patients using recombinant FSH (rFSH)/human FSH or rFSH + human menopausal gonadotropin (HMG) in various flexible protocols. In the luteal phase long gonadotrophin-releasing hormone agonist (GnRH-a) protocol, patients were administered a 0.1-mg triptorelin daily injection for 14 days or a single 1.3/1.8-mg triptorelin injection during the midterm-luteal phase of the previous menstrual period, followed by recombinant FSH (GONAL-f; Merck Serono, Geneva, Switzerland/Purigon; Organon, Oss, The Netherlands)/human FSH (Livzon Pharmaceutical Group, Zhuhai, China) with or without hMG (Livzon Pharmaceutical Group). In the follicular phase long gonadotrophin-releasing hormone agonist (GnRH-a) protocol, patients underwent pituitary down-regulation with 3.75-mg of triptorelin acetate or leuprorelin acetate on the first day of the cycle, followed by rFSH or in combination with HMG 28–35 days later. In the short protocol cycle, patient received GnRH-a from the 2nd day of the menstrual cycle onward, then rFSH or in combination with HMG on the 3rd day. Patients were started with rFSH treatment on the 2nd day of the cycle by once-daily injection in the antagonist protocol. Follicle development was monitored by vaginal ultrasound. After 4–5 days of stimulation, the antagonist (cetrorelix acetate or ganirelix acetate) was administered once daily. The rFSH dose was adjusted according to the individual ovarian response, which was assessed by daily ultrasound examinations. The antagonist continued up to and including the day of human chorionic gonadotropin (hCG) administration.

In the mild stimulation protocol, patients were started on clomiphene citrate (50–100 mg) on day 2–6 of the cycle once daily and rFSH/HMG (150–225IU/day) injection from day 3. Follicle development was monitored by vaginal ultrasound on day 8 of the cycle. An antagonist (cetrorelix acetate or ganirelix acetate) was administered once daily. The rFSH/HMG dose was adjusted according to the ovarian response, which was assessed by daily ultrasound examination. In all treatment protocols, when at least two leading follicles reached 18 mm in size, ovulation was triggered by administering 250 μg of r-hCG (Merck Serono S.p.A), and ovum collection was subsequently performed 34–38 h later.

The intervention in the GH group included the subcutaneous injection of 2 IU of human recombinant GH (Jintropin, Changchun, China) per day for 4 weeks before COS, then 4 IU/d of GH beginning on the initial day of gonadotrophin administration until the day of hCG injection. GH administration and dose may be adjusted for patient age and BMI at the discretion of each clinician.

Luteal support was used as follows. In the fresh cycles, patients inserted 8% progesterone sustained-release vaginal gel [90 mg vaginally (crinone)] daily on the day of oocyte pick-up until the day of HCG assay 14 days after embryo transfer. In the frozen cycles, our patients were divided into two groups (artificial and natural cycles). Follicle grow-up was monitored with vaginal B ultrasound in the natural cycle group to determine the day of ovulation, followed by the daily administration of 20 mg of

dydrogesterone orally on the 3rd day after ovulation until day 14. In the artificial group, 6–8 mg of estradiol valerate (E2V) daily were administrated from the 3rd day of the cycle, followed by the daily insertion of 8% crinone vaginally on day 14, then adding 20 mg of dydrogesterone twice a day on day 18. The embryo transfer was done on the day of dydrogesterone administration in the nature or artificial cycle, and the blastocyst transfer was done on the 3rd day after dydrogesterone administration in both groups.

Laboratory Protocol

IVF and ICSI were performed according to routine laboratory insemination procedures on the day of oocyte retrieval. The presence of two pronuclei was observed 17–19 h after insemination or injection, and the zygotes were then cultured in 25-ml droplets of pre-equilibrated G1-Plus (Vitrolife, Gothenburg, Sweden). Embryo morphology was evaluated with respect to cell number, fragmentation, and symmetry 68–72 h after insemination. Generally, good quality embryos (5–10 cell embryos with < 20% fragmentation) was transferred on day 3 or frozen by vitrification on this stage. The remaining good quality embryos were placed in G2-plus (Vitrolife), until they reached the blastocyst stage. Blastocysts reaching the expanded or hatching stage and earning a score above grade 4CC (inner cell mass/trophectoderm) according to the Gardner criteria (12) were transferred or cryopreserved by vitrification.

Vitrification and Warming Procedures

The expanded blastocysts collapsed after artificial shrinkage and were vitrified and warmed. Briefly, the blastocysts were equilibrated in 7.5% (v/v) dimethyl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO, USA) and 7.5% (v/v) ethylene glycol (EG; Sigma Chemical Co.) at 37°C for 2 min and placed in 15% DMSO, 15% EG and 0.65 mol/l sucrose for 30 s. During this period, one blastocyst was placed on the Cryotop strip (Kitazato, Fuji, Japan), which was then quickly plunged into liquid nitrogen. For warming, the Cryotop was quickly placed in 0.33 mol/l sucrose at 37°C. After 2 min, the blastocysts were transferred into 0.2 mol/l sucrose for 3 min and in HEPES-buffered medium for 5 min. Subsequently, the blastocysts were cultured in G2-plus medium for 2 h to evaluate the quality. Blastocysts with good survival (less than one-half of the blastocysts showing signs of damage) and showing re-expansion were transferred. The DMSO–EG–sucrose system as cryoprotectants was also used for day 3 embryo freezing and warming.

Definition of Outcomes

The main outcome was clinical pregnancy rate per transfer cycle. The secondary outcomes were as follows: the number of retrieved oocytes, two pronuclei (PN) zygote, day 3 available embryo; implantation rate, early miscarriage rate (<12 weeks) and live birth rate. An embryo was defined as an available embryo on day 3 if the embryo had ≥ 5 cells and included < 20% anucleated fragments. The implantation rate was calculated as the ratio of the number of gestational sacs-to-the number of embryos transferred. A clinical pregnancy was diagnosed when a gestational sac was demonstrated by transvaginal ultrasound scan 4 weeks after embryo transfer. Live birth rate was calculated at a ratio of live births-to-the number of embryos transferred minus those lost to follow-up.

Statistical Analysis

Sample size calculation was performed with Power Analysis and Sample Size software (PASS). In our database the clinical pregnancy rate was 34% in the GH group and 30% in the control group; the clinical pregnancy rate increased by 10%, which was clinically significant (13, 14). The type I error was set at 0.05, and the type II error was set at 0.2. Maternal age was matched at a 1:1 ratio. After testing, the sample size of the study and control groups was at least 138 cases. All statistical analyses were performed with the Statistical Package for the Social Sciences software (version 17.0; SPSS, Inc., Chicago, IL, USA). Continuous variables were compared using analysis of variance and categorical variables were evaluated with a chi-square test. All tests were two-sided, and a $P < 0.05$ was considered statistically significant.

Ethical Approval

This retrospective cohort study was approved by the Ethics Committee of Reproductive Medicine at Peking University Third Hospital on 16 AUG 2019; the reference number is 2019SZ-062.

RESULTS

Three hundred seventy-six frozen-thawed cycles were recruited into the GH or control group at a ratio of 1:1. The mean age of women was 36 years. The distribution of ages for women was similar between the two groups (p=0.838). The AMH (0.69 ± 0.31 *vs*. 0.63 ± 0.32; p = 0.065) and AFC (4.10 ± 1.63 *vs*. 3.96 ± 1.72; p = 0.423) were comparable between the two groups. Patient characteristics, including parental BMI, type of infertility, causes of infertility, infertility duration, and parity were not different across arms of the study (**Table 1**). The COS protocol was significantly different between the arms of the study (p=0.001). The number of oocytes (7.13 ± 3.93 *vs*. 5.89 ± 3.33; p = 0.001), two PN zygotes (4.66 ± 2.76 *vs*. 3.99 ± 2.31; p = 0.011), and day 3 available embryos (3.86 ± 2.62 *vs*. 3.26 ± 2.04; p=0.014) obtained in the GH group, were significantly higher than the control group (**Table 2**). There was no significant difference between the two groups with respect to the number of frozen embryos (2.29 ± 2.11 *vs*. 1.92 ± 1.55; p = 0.055) and cycles with a fresh transfer (47.9 *vs*. 45.2%; p = 0.605; **Table 2**).

A total of 538 embryos were thawed. The survival rate was comparable between the GH and control groups (94.8% *vs*. 93.3%; p=0.440). The proportion of hormone replacement and natural protocols was not different across the arms of the study. Endometrial thickness (9.68 mm ± 1.61 *vs*. 9.80 mm ± 1.69; p = 0.529), day 3 or blastocyst transfer (p=1.000), and the number of embryos transferred (1.25 ± 0.45 *vs*. 1.24 ± 0.44; p = 0.747) were comparable across the arms of the study (**Table 3**).

TABLE 1 | Characteristics of patients.

	GH (n=188)	Control (n=188)	P value
Maternal age (years)	36.06 ± 4.60	36.06 ± 4.48	1.000
<35	59 (31.4%)	62 (33.0%)	0.838
35–40	77 (41.0%)	79 (42.0%)	
≥40	52 (27.7%)	47 (25.0%)	
Paternal age (years)	37.02 ± 5.21	37.41 ± 6.30	0.515
Maternal BMI (kg/m^2)	23.02 ± 3.30	22.80 ± 3.19	0.498
Paternal BMI (kg/m^2)	25.87 ± 3.77	25.78 ± 4.04	0.825
Semen density (million)/ml	66.49 ± 51.97	67.62 ± 53.30	0.842
Infertility duration (years)	4.61 ± 3.63	4.45 ± 3.80	0.681
Primary infertility (%)	81 (43.1%)	71 (37.8%)	0.426
Nulliparous (%)	162 (86.2%)	159 (84.6%)	0.662
Main infertility cause (%)			0.761
Female	113 (60.1%)	121 (64.4%)	
Male	8 (4.3%)	5 (2.7%)	
Mixed	65 (34.6%)	60 (31.9%)	
Unexplained	2 (1.1%)	2 (1.1%)	
Basal hormone			
FSH (mIU/ml)	8.86 ± 5.06	9.05 ± 3.62	0.698
E_2 (pmol/L)	316.53 ± 744.31	291.94 ± 455.96	0.718
LH (mIU/ml)	3.43 ± 2.17	3.94 ± 2.79	0.058
AMH (ng/ml)	0.69 ± 0.31	0.63 ± 0.32	0.065
AFC	4.10 ± 1.63	3.96 ± 1.72	0.423

GH, growth hormone; BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; E2, estradiol; AMH, anti-Mullerian hormone; AFC, antral follicle count; ET, embryo transfer.

TABLE 2 | Controlled ovarian stimulation (COS) protocol and laboratory parameters in fresh cycle.

	GH (n=188)	Control (n=188)	P value
Protocol (%)			
Luteal phase long	21 (11.2%)	9 (4.8%)	0.001
Follicular phase long	39 (20.7%)	29 (15.4%)	
Short protocol	7 (3.7%)	9 (4.8%)	
Antagonist protocol	73 (38.8%)	85 (45.2%)	
Mini-stimulation	28 (14.9%)	50 (26.6%)	
No of collected oocyte	7.13 ± 3.93	5.89 ± 3.33	0.001
No of 2PN zygote	4.66 ± 2.76	3.99 ± 2.31	0.011
No of available embryo	3.86 ± 2.62	3.26 ± 2.04	0.014
No of frozen embryo	2.29 ± 2.11	1.92 ± 1.55	0.055
Utilization rate	57.6%	57.9%	0.892
Fresh ET	90 (47.9%)	85 (45.2%)	0.605

PN, pronuclei; ET, embryo transfer. Utilization rate = percentage of embryos suitable for transfer or freezing.

TABLE 3 | Characteristics of frozen-thawed cycle treatment.

	GH (n=188)	Control (n=188)	P value
Protocol (%)			0.916
Hormone replacement	74 (39.4%)	75 (39.9%)	
natural	114 (60.6%)	113 (60.1%)	
Endometrial thickness (mm)	9.68 ± 1.61	9.80 ± 1.69	0.529
Thawed embryos	271	269	
Surviving embryos	257 (94.8%)	249 (93.3%)	0.440
Thawed ET (%)	185 (98.4%)	184 (97.9%)	1.000
D3 ET	55 (29.3%)	56 (29.8%)	0.883
D5 ET	130 (69.2%)	128 (68.1%)	
No. of transferred embryos	1.25 ± 0.45	1.24 ± 0.44	0.747

GH, growth hormone; ET, embryo transfer.

As shown in **Table 4**, there was no significant difference in the clinical pregnancy rate (30.3% *vs.* 31.0%; p = 0.883), implantation rate (25.3% *vs.* 26.2%; p = 0.829), early abortion rates (16.1% *vs.* 15.8%; p = 0.967) and live birth rate (20.6% *vs.* 20.8%; p=0.980). The proportion of cycles with remaining frozen embryos was significantly higher in the GH group than the control group (36.2% *vs.* 26.6%; p = 0.045). Clinical outcomes were also comparable in the POR subgroup stratified by maternal age (**Table 5**) and the COS protocol (**Table 6**). However, the subgroup with good quality blastocyst transfer (≥4BB) demonstrated an improvement in the clinical pregnancy (46.8% *vs.* 32.1%; p = 0.075), early miscarriage (10.3% *vs.* 20.0%; p = 0.449), and live birth rates (35.7% *vs.* 18.9%; p = 0.031) following GH co-treatment (**Table 7**).

DISCUSSION

In agreement with previous findings reviewed by Yovich et al. (15), the embryogenesis parameters were significantly increased in PORs administered GH. Moreover, the current study showed

TABLE 4 | Clinical outcomes of frozen-thawed cycles.

	GH (n=185)	Control (n=184)	P value
Clinical pregnancy rate/ET (%)	56 (30.3%)	57 (31.0%)	0.883
D3ET	15/55 (27.3%)	17/56 (33.3%)	0.720
SET	1/13 (7.7%)	3/15 (9.1%)	0.600
DET	14/42 (33.3%)	14/41 (34.1%)	0.938
D5ET	41/130 (31.5%)	40/128 (31.3%)	0.960
SET	39/124 (31.5%)	40/124 (32.3%)	0.892
DET	2/6 (33.3%)	0/4 (0.00%)	0.467
Implantation rate/ET (%)	59/233 (25.3%)	60/229 (26.2%)	0.829
Early miscarriage (<12weeks)/CP (%)	9 (16.1%)	9 (15.8%)	0.967
Lost to follow-up at birth	10	11	
Live births	36 (20.6%)	36 (20.8%)	0.980
Cycles with embryo surplus	68/188 (36.2%)	50/188 (26.6%)	0.045

GH, growth hormone; SET, single embryo transfer; DET, double embryo transfer; ET, embryo transfer; CP, clinical pregnancy.

TABLE 5 | Clinical outcomes of frozen-thawed cycles stratified by maternal age.

	GH (n=185)	Control (n=184)	P value
Clinical pregnancy rate/ET (%)			
<35	26/59 (44.1%)	25/62 (40.3%)	0.677
35–<40	25/76 (32.9%)	26/76 (34.2%)	0.864
≥40	8/50 (16.0%)	9/46 (19.6%)	0.648
Early miscarriage rate/CP (%)			
<35	4/26 (15.4%)	4/25 (16.0%)	1.000
35–<40	7/25 (26.9%)	3/26 (11.5%)	0.173
≥40	0/8 (0.0%)	4/9 (44.4%)	0.082
Live birth/ET (%)			
<35	19/56 (33.9%)	13/54 (24.1%)	0.255
35–40	13/73 (17.8%)	19/73 (26.0%)	0.230
≥40	4/46 (8.7%)	4/46 (8.7%)	1.000

GH, growth hormone; ET, embryo transfer; CP, clinical pregnancy; Live birth rate = live birth/(ET-lost follow).

TABLE 6 | Clinical outcomes of frozen-thawed cycles stratified by stimulation protocol.

	GH (n=185)	Control (n=184)	P value
Clinical pregnancy rate/ET (%)			
Follicular phase long	12/39 (30.8%)	10/29 (34.5%)	0.746
antagonist	22/71 (31.0%)	30/82 (36.6%)	0.466
Mini-stimulation	7/28 (25.0%)	14/49 (28.6%)	0.420
Early miscarriage (<12 week) rate/ CP (%)			
Follicular phase long	4/12 (33.3%)	1/10 (10.0%)	0.323
antagonist	4/22 (18.2%)	8/30 (26.7%)	0.473
Mini-stimulation	1/7 (14.3%)	2/14 (14.3%)	1.000
Live birth rate/ET			
Follicular phase long	4/36 (11.1%)	7/27 (25.9%)	0.182
antagonist	14/68 (20.6%)	16/77 (20.8%)	0.977
Mini-stimulation	6/28 (21.4%)	8/46 (17.4%)	0.667

GH, growth hormone; ET, embryo transfer; CP, clinical pregnancy.

TABLE 7 | Clinical outcomes of frozen-thawed cycles stratified by embryo quality.

	GH (n=185)	Control (n=184)	P value
Clinical pregnancy rate/ET (%)			
Day 3 (≥8 cell)	13/35 (37.1%)	15/43 (34.9%)	0.836
Blastocyst (≥4BB)	29/62 (46.8%)	25/78 (32.1%)	0.075
Early miscarriage (<12 week) rate/ CP (%)			
Day 3 (≥8 cell)	3/13 (23.1%)	1/15 (6.7%)	0.311
Blastocyst (≥4BB)	3/29 (10.3%)	5/25 (20.0%)	0.449
Live birth rate/ET			
Day 3 (≥8 cell)	8/34 (23.5%)	9/38 (23.7%)	0.988
Blastocyst (≥4BB)	20/56 (35.7%)	14/74 (18.9%)	0.031

GH, growth hormone; ET, embryo transfer; CP, clinical pregnancy.

that GH adjuvant therapy may improve the live birth rate for a POR subgroup with good quality blastocyst transfer (≥4BB) in frozen-thawed cycles.

Based on a previous study, the maternal age at the time of oocyte retrieval and ovarian reserve significantly affect the clinical pregnancy rate in frozen-thawed cycles (16). Therefore, we accurately matched maternal age in fresh cycles, and excluded the main confounding factors between the study and control groups. The AMH and AFC values were below the cut-off values, as suggested by other studies (17, 18), which have high discriminatory abilities between expected and unexpected PORs (19). The ovarian stimulation protocol was significantly different between the two groups. however, neither protocol was superior with respect to pregnancy outcomes with PORs (20, 21). The advantage of this study was that patient profile and cycle treatment baselines were comparable between the arms of the study. The major limitation of the current study, however, was the retrospective design. Because there is no consensus on GH administration in clinical practice among clinicians, the GH administration protocol and injection dose may be variable with patient profile and affordability.

The current study indicated that GH administration significantly increased the number of oocytes retrieved, 2PN zygotes, and day 3 available embryos, while the number of frozen embryos was also greater in the GH group. Although there is an opinion that a higher oocyte number can be translated to a higher probability of clinical pregnancies and live births, this viewpoint has been contradicted by new evidence, suggesting that a high oocyte yield does not improve the success rate in single frozen-thawed transfers (22). In the current study, GH administration not only increased the oocyte number, but also improved the intrinsic quality of the embryos. The improvement in oocyte and embryo quality resulted in a higher live birth rate in the first frozen-thawed embryo transfer in a subgroup of PORs. Interestingly, the beneficial effect of GH administration on the pregnancy and live birth rates was found in blastocyst transfers (≥4BB), but not in day 3 embryos (≥8 cell). In addition, there were more cycles with surplus frozen embryos in the GH group than the control group; this finding may result in a higher probability of cumulative pregnancy and live birth rates.

Yovich and Stanger suggested that GH co-treatment significantly improves the clinical pregnancy rate per fresh transfer per frozen-thawed embryos derived from GH cycles (11). A recent study from the same center showed that poor-prognosis patients receiving GH co-treatment during stimulation cycles have similar live birth rates with good prognosis patients in the first frozen-thawed cycle, and demonstrated a beneficial effect of GH administration on the live birth rate [OR 2.71; (1.14–6.46)] in frozen-thawed cycles (10). These data from a single center uniquely showed that the effect of GH is directed at oocytes and subsequent embryo quality. Our study, to some extent, is in agreement with their findings (10, 11). In addition, it has been reported that GH supplementation may increase the pregnancy and implantation rates, and decrease the miscarriage rate in older women (14, 23, 24). Lan et al. reported that GH improves endometrial imaging during ultrasonography and enhances endometrial receptivity in women older than 40 years old (14). Other observational studies reported that GH co-treatment increased the probability of pregnancy in fresh cycles for POR; however, this study was limited by a small sample size (25–27). The beneficial effect of GH administration on PORs of advanced age was likely due to endometrial image improvement.

Several randomized controlled trials (RCTs) were designed to evaluate the effectiveness of GH supplementation. Bassiouny et al. reported similar pregnancy and live birth rates in two arms of a study (141 PORs) (28). Similarly, Dakhly et al. also failed to detect a beneficial impact of GH addition on live births (240 PORs fulfilling Bologna) (29). Recently, live birth, *in vitro* fertilization and GH treatment (LIGHT) with a double-blind design study reported no improvement in oocyte number, and pregnancy and live birth rates (130 PORs) (30). It is challenging to conduct large-scale RCTs on PORs. Systematic reviews compiling RCTs suggested that GH supplementation may improve the clinical pregnancy (31–33) and live birth rates in PORs (32, 33). It is reported that at least 150 participants per study group are required to detect clinically important differences in reproductive outcomes in PORs (34). In the current study, 376 PORs fulfilling the inclusion criteria were

large enough to detect a significant difference in clinical pregnancy and live birth rates.

In the current study, GH co-treatment was shown to be beneficial to the POR subgroup with good quality blastocyst transfers in terms of live birth rate. The difference in live births should be cautiously interpreted because the sample size in this subgroup was relatively small. In the future, true efficacy of GH supplementation on pregnancy and live birth rates should be verified by a large-scale multi-center RCT.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Reproductive Medicine, Peking University Third Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceived and designed the study: JQ. Coordinated data collection: PL, RL, YW. Analyzed the data: JZ, YW. Drafted the manuscript: JZ. All authors contributed to the article and approved the submitted version.

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REVIEW
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Prospects of Germline Nuclear Transfer in Women With Diminished Ovarian Reserve

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Diminished ovarian reserve (DOR) is associated with a reduced quantity and quality of the retrieved oocytes, usually leading to poor reproductive outcomes which remain a great challenge for assisted reproduction technology (ART). Women with DOR often have to seek for oocyte donation, precluding genetically related offspring. Germline nuclear transfer (NT) is a novel technology in ART that involves the transfer of the nuclear genome from an affected oocyte/zygote of the patient to the cytoplast of an enucleated donor oocyte/zygote. Therefore, it offers opportunities for the generation of genetically related embryos. Currently, although NT is clinically applied only in women with serious mitochondrial DNA disorders, this technology has also been proposed to overcome certain forms of female infertility, such as advanced maternal age and embryo developmental arrest. In this review, we are proposing the NT technology as a future treatment option for DOR patients. Strikingly, the application of different NT strategies will result in an increase of the total number of available reconstituted embryos for DOR patients.

Keywords: diminished ovarian reserve, poor ovarian response, oocyte quality, germline nuclear transfer, spindle transfer, polar body transfer

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INTRODUCTION

Ovarian reserve refers to the number of primordial follicles residing in the ovary and determines the reproductive lifespan of a woman (1). The number of follicles is predetermined at birth, with female ovaries containing approximately 1,000,000 immature oocytes (2). After birth, this number progressively decreases due to apoptosis and cyclic recruitment of follicles in every menstrual cycle, until menopause, after which the number of follicles will have been reduced to about 1,000 (3). Reproductive efficiency decreases over the years due to this follicular loss, but also as a result of the decreasing quality of the remaining oocytes due to reported increased aneuploidy rates, low fertilization competence, compromised mitochondrial function and higher spontaneous abortion risk (1, 4, 5). The efficiency of the female reproductive outcome reaches a peak in the mid-20s and starts declining slowly, dropping dramatically after 37 years of age (4, 6).

Diminished ovarian reserve (DOR) is characterized by a decrease in the quantity of the ovarian follicular reserve and is mostly attributed to the advanced age of the patient, but also to non-

physiological parameters, such as genetic background, surgical interventions, therapies for cancer treatment (7–13). Increased levels of Follicle stimulating hormone (FSH >10 mIU/ml), low Antral follicle counts (AFC <5–7 follicles), and decreased Anti-Mullerian hormone (AMH) levels (<0.5–1.1 ng/ml) are markers for DOR diagnosis (14). Patients with DOR have an occurrence of 31% in ART (15) cycles and are considered as challenging as they usually display poor ovarian response (POR), leading to a lower number of retrieved oocytes and subsequently fewer embryos available for transfer, with resulting poor pregnancy and life birth rates (LBR) (16). Bologna and POSEIDON (Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number) criteria are being used as means to identify and treat these patients (17–19).

The incidence of POR patients in the ART setting might vary between 6 and 35% (20) while it can be over 50% in women over their forties (21). Several treatment strategies for these patients are being investigated as means to increase the oocyte yield and improve the LBR. These approaches include changes in the type and dose of gonadotropins, stimulation protocols, the use of adjuvants, double stimulation cycles and the manipulation of ovarian tissue for primordial follicle activation. The choice of the appropriate treatment depends on the characteristics of the patient (22–30). Despite the current attempts to improve the poor ovarian response to hormonal stimulation (18, 31, 32), current strategies remain experimental or inconclusive. Child adoption or oocyte donation remains the only options for some patients to fulfil a child wish (33, 34).

DOR in aged women has also been associated with concomitant poor oocyte quality (35–37). Poor oocyte quality is linked to cytoplasmic insufficiency, as several cytoplasmic factors, including mitochondria, metabolites, maternal RNAs and proteins, are important regulators of oocyte and embryo competence (38). In comparison with the quantitative decrease in oocyte numbers, oocyte quality is not easy to assess. In the ART setting, oocyte quality has been linked to morphological features of the oocytes, embryo arrest, blastulation, implantation, pregnancy, miscarriage, and euploidy rates. The ultimate marker is the live birth of a healthy offspring (36). Oocyte quality does not seem to be affected in young DOR patients, when compared to age matched groups with a normal ovarian reserve (37, 39, 40). On the contrary, DOR patients of advanced maternal age have significantly lower chances for a clinical pregnancy when compared to younger DOR patients, higher miscarriage rates and lower high-quality embryos, suggesting poor oocyte quality (40).

Current approaches for assisting patients with DOR and POR could be expanded with the novel germline nuclear transfer (NT) technology. NT offers the possibility to transfer the genetic material of a patient's oocyte/zygote with compromised cytoplasm to the cytoplasm of an enucleated oocyte/zygote of a healthy donor. This technology enables the generation of embryos, to which both parents have contributed genetically (41). NT is currently clinically applied in a strict subset of patients suffering from mitochondrial DNA (mtDNA) disorders, aiming to overcome maternal mutant mtDNA transmission to the next generation. Besides, increasing interest has been shown in the application of NT to overcome certain types of female-related infertility (42), which is supported by some promising studies in animal models (42, 43), but still, peer-reviewed reports in human patients remain scarce. The rationale of this treatment is that by transferring the nuclear DNA from an oocyte with inferior quality cytoplasm to an oocyte with more competent cytoplasm, like the ones of young, fertile women, could potentially improve embryo development. In this review, we propose the NT technology as a possible novel treatment method for DOR patients in order to increase the number or/and quality of the retrieved embryos.

DIFFERENT NUCLEAR TRANSFER TECHNIQUES

For normal fertilization to occur, an oocyte needs to be at the correct maturation state, both cytoplasmic as nuclear. Before ovulation, oocytes are arrested in meiotic prophase I. They are diploid, having both paternal and maternal genomes, and DNA resides in the nucleus (germinal vesicle (GV)). Following meiotic maturation, oocytes arrest in the metaphase of meiosis II (MII oocyte). Chromosomes are aligned on the spindle, while the oocyte extrudes the first Polar body (PB1) containing the homologous chromosomes. At this stage, the oocyte can be fertilized by the sperm. Following fertilization, the oocyte completes Meiosis II, extruding first a second Polar body (PB2) and forming the maternal and paternal pronuclei, generating the zygote. The PB2 contains the haploid sister chromatids of the maternal pronucleus (44). The NT technology can be performed at different stages of oocyte maturation: Germinal vesicle transfer (GVT), Spindle transfer (ST) or Polar body 1 transfer (PB1T) or at the zygote stage: Pronuclear transfer (PNT) or Polar body 2 transfer (PB2T).

During GVT, the nucleus of a GV oocyte is being transferred in an enucleated GV donor oocyte (**Figure 1**). The reconstructed oocyte needs to undergo *in vitro* maturation before it can be fertilized by the sperm (45). In ST, the spindle-chromosome complex (containing the DNA) of an MII oocyte is transferred to an enucleated (DNA free) MII donor oocyte which is subsequently fertilized by the sperm of the patient's partner (46) (**Figure 1**). At the MII stage, also PB1T may occur. PB1T includes the transfer of the first polar body into an enucleated MII oocyte, followed by fertilization (**Figure 1**). In PNT, the pronuclei from the patient's zygote can be transferred in the cytoplasm of an enucleated donor zygote that will serve as a recipient (46) (**Figure 1**). At this stage, also PB2T may occur, which involves the transfer of PB2 in an enucleated donor oocyte containing only the male pronucleus from the partner's sperm. The paternal pronucleus can be obtained in two ways: a. Fertilization of the donor oocyte with sperm and subsequent removal of the maternal pronucleus (46, 47) or b. Enucleation of a mature oocyte and subsequent fertilization by the partner's sperm. In this case, only one pronucleus is formed. Polar body 2 can be transferred in the zygote with the male pronucleus and

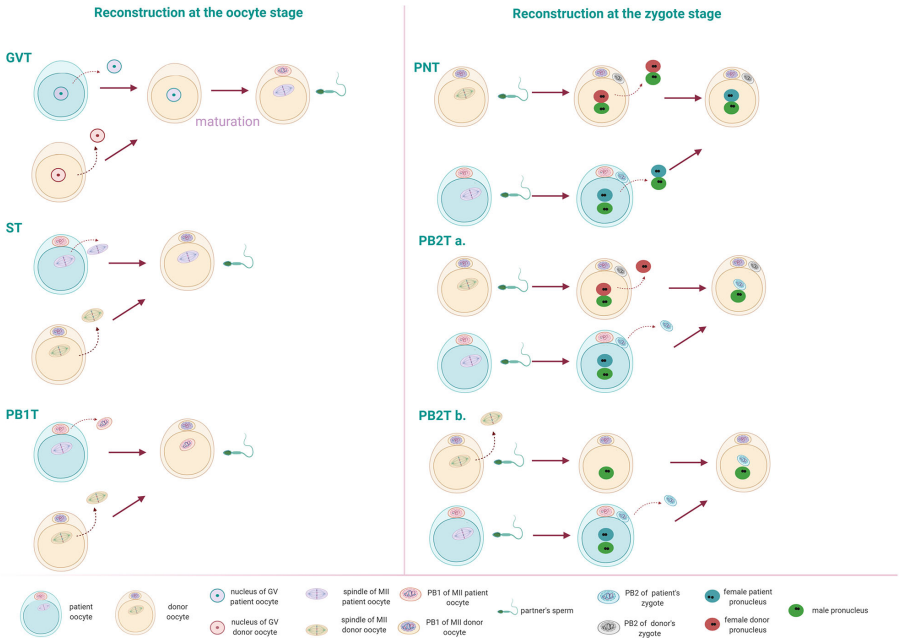

FIGURE 1 | Different nuclear transfer (NT) techniques can occur at the oocyte or the zygote stage. Reconstruction at the oocyte stage: Germinal vesicle transfer (GVT): the nucleus is transferred in an enucleated GV oocyte. Following *in vitro* maturation, the reconstructed oocyte can be fertilized by the patient's partner sperm. Spindle transfer (ST): The spindle from a mature oocyte (MII) is transferred to the enucleated MII donor oocyte. Polar body 1 transfer (PB1T): The first polar body from an MII oocyte is transferred to an enucleated MII oocyte of a donor. Reconstruction at the zygote stage: Pronuclear transfer (PNT): the pronuclei from a fertilized oocyte are transferred to the enucleated donor zygote. Polar body 2 transfer (PB2T): The second polar body of a fertilized oocyte is transferred to a zygote containing the male pronucleus of the patient's partner. The paternal pronucleus can occur in two ways: (a) by fertilization of a donor's oocyte. Following fertilization, the male and female pronuclei form. The female pronucleus of the donor can be removed and replaced by the second polar body of the patient's MII oocyte. (b) The donor's MII oocyte is enucleated and injected with the partner's sperm. Following the formation of the male pronucleus, the second polar body from a patient's zygote can be transferred.

result in an embryo (48). The latter approach is the most appropriate in human, as it is hard to distinguish between male and female pronucleus (**Figure 1**).

NT techniques have been tested in several species, including non-human primates. Mice have been used as models in the vast majority of published papers to evaluate the efficacy of this technology due to their fast reproduction, easier manipulations, and abundancy of gamete cells. Another benefit of making use of these species is some conserved similarities with humans regarding the events of gametogenesis and early embryonic development (49). Following fertilization, the formation of the two pronuclei in the mammalian species signals the zygote formation. Zygote formation is followed by consecutive divisions of the newly formed embryo. Embryonic genome activation (EGA) is an important event that occurs after the first cell divisions, and it differs according to the species: in mouse: two-cell stage and in human between four and eight cells (50). Before EGA, the maternal proteins and mRNAS already present in the oocyte direct almost exclusively the first events of fertilization and embryonic development. Following EGA, embryonic cells compact and polarize to form two distinct cellular populations at the blastocyst stage. In mammals, embryos will implant at this stage, following the release of the blastocyst from the zona pellucida. Besides the similarities at these early embryonic stages, mice do display important differences when compared to larger mammals and humans. Embryonic developmental events occur faster in the mouse species. The events of EGA, cell division, compaction, blastulation, and implantation occur much faster in mice compared to human (49). Furthermore, mouse embryos have a prominent good embryonic development (reaching approximately 80% blastocyst rate) when cultured *in vitro*, when compared with larger mammalian species, including humans. In addition, meiotic errors in mouse oocytes are less evident than in human, with an estimated incidence of 1 and 5–20% respectively. Moreover, human embryos appear to have high numbers of mosaicism and aneuploidy rates than mouse embryos (49). Although mammalian species have served as good models to study the efficacy and safety of NT, species-specific differences usually demand a fine-tuning of the technology in the human species but also careful interpretation of the results.

For the abovementioned reasons, optimization of the technique in human oocytes was necessary before consideration of any future clinical application. These studies have focused on the efficiency of the NT technology using human oocytes regarding reconstruction and blastocyst rates and the amount of cytoplasmic carry-over from the oocyte that serves as a nuclear donor. A previous study by Craven et al. (51),

performed PNT using human abnormally fertilized zygotes (1PN and 3PN). Reconstruction was successful, with less than 2% mitochondrial DNA carry-over from the nuclear donor. Nevertheless, a very low blastocyst formation rate was observed (8.3%) in comparison to unmanipulated controls (51). The PNT procedure was further optimized using normally fertilized zygotes and by refining the timing of the procedure. Early PNT, performed at 8 h post-ICSI was shown to be beneficial upon late PNT at 16 h post-ICSI. This adapted methodology was shown to significantly increase the blastocyst rates of the reconstructed zygotes, to a level of the non-manipulated controls (52). The first study to perform ST in human MII oocytes was published by Tachibana et al. (53), which confirmed the feasibility of the technique in human oocytes. Blastocyst rate in reconstructed oocytes following normal fertilization showed similar results with control fertilized oocytes (62 *vs* 76%). Human embryonic stem cells (hESCs) were also derived from the reconstructed embryos, carrying low levels of mtDNA from the nuclear donor (53). While ST and PNT have been mostly studied in the human, only one study has been reported making use of the PB1T strategy. Reconstructed PB1T oocytes were capable of normal fertilization, and PB1T zygotes developed to the blastocyst stage, but yet in a lower rate (42%) compared to the control group (75%) (54). PB2T studies in the human have been scarcely described, possibly due to the difficulty of distinguishing between male and female PNs (47). Mouse models using this technology have proven very promising but nevertheless, in the mouse it is easy to distinguish between female and male pronucleus, due to their evident size difference (47). The novel PB2T described by Tang et al. (48), optimized the use of PB2T in human oocytes and proved its efficacy. GVT remains challenging, as oocyte *in vitro* maturation is not yet optimized. In a mouse model, GVT oocytes were able to undergo maturation and cleave, but all embryos arrested before the blastocyst stage (55). In human, more studies are needed to improve the efficiency of the technique before it could be considered for clinical application, as still, *in vitro* maturated oocytes are inferior compared to their *in vivo* counterparts (56).

APPLICATIONS OF GERMLINE NUCLEAR TRANSFER

Nuclear Transfer for Mitochondrial Diseases

NT techniques have initially been proposed to prevent the transmission of mtDNA diseases from the mother to the offspring. Mitochondrial disorders are reported to affect one in 5,000 individuals and are attributed to mutations or deletions in the mitochondrial DNA (mtDNA). Mitochondria are semi-autonomous organelles, important for the energy production and metabolism of the cell. They hold their own genome (mitochondrial DNA, mtDNA) in a variety of copies, coding for 37 genes. Other genes important for mitochondrial functions are encoded by the nuclear genome of the cell. Thus, mitochondrial function is under dual genetic control of both the mitochondrial and nuclear genome (57). Mitochondria are exclusively inherited only from the mother (58, 59) with a few rare exceptions being reported recently of possible paternal inheritance (60). Oocyte mtDNA mutations either reside in a homoplasmic state, with all of the copies carrying the mutation, or in a heteroplasmic state, presenting a mixture of mtDNA mutated and wild-type copies. The degree at which heteroplasmy occurs can differ between cells and tissues of one individual but can also shift in between generations. This can be attributed to the processes occurring during the formation of the female germline. At the stage of primordial germ cell formation, the number of mtDNA copies decreases significantly, which is a phenomenon designated as the mitochondrial genetic bottleneck (61). For women carrying heteroplasmic mtDNA mutations, this process can trigger a shift in heteroplasmy levels in the produced oocytes and makes it therefore difficult to predict the mutational load (number of affected mtDNA copies) in the corresponding generated embryo. Until now, there are no treatments available to eliminate mitochondrial diseases, only ways of prevention (62).

Pre-implantation genetic testing (PGT) has been used to determine the level of pathogenetic mtDNA copies in *in vitro* generated embryos in order to select mutation-free or mutation-low embryos which will not be affected by a mitochondrial disease (63). The mutational load should be less than 18% for an embryo to be considered safe for transfer, as calculated by a meta-analysis study by Hellebrekers et al., regardless the type of mtDNA mutation (64). Nevertheless, PGT might have diagnostic limitations, as embryonic mitochondria may shift their heteroplasmy levels during cell division, and mitochondrial mutations may be favored in response to environmental influences over wild type copies. In addition, patients carrying homoplasmic DNA mutations cannot be helped by PGT (65).

The NT technology can be beneficial for both patients carrying homoplasmic mutations, as well as for patients carrying heteroplasmic mutations for which no embryos with mtDNA mutational levels below 18% can be identified by PGT. By transferring the nuclear genetic material of the patient's diseased oocyte/zygote to an enucleated, donated oocyte/zygote, containing healthy mtDNA copies, the reconstructed embryo is genetically related to both parents, with mtDNA being associated to the oocyte or zygote donor (66). During NT, it is inevitable that a minimal amount of cytoplasm is being transferred along, so a certain amount of the patient's mtDNA copies will be present in the reconstructed NT embryos as well, which is known as the carry-over (67).

NT is a quite controversial topic in the field of ART, as it remains a new technology, and little is known about the effect on the health of the offspring. Studies in animal models demonstrated the feasibility of this technique and the potential to prevent mitochondrial diseases (47, 55, 68). A number of preclinical studies in human have also reported the carry-over of the patient's oocyte to the donor's cytoplasm and also verified the efficacy of PNT, ST, and PBT (52–54, 69). The first live birth in human was published in 2017, where the NT technology was

used to overcome the transmission of Leigh syndrome, a mitochondrial disease, to the offspring (70). The patient had a long history of undiagnosed pregnancy losses and offspring death due to the disease. Following ST of the patient's oocytes into enucleated oocytes of a donor and subsequent fertilization with the partner's sperm, a healthy baby was born. The offspring was carrying low levels of mutant maternal mtDNA (2.36–9.23% in the tested tissues), indicating both the efficacy of the technique to prevent mitochondrial diseases, as well as the occurrence of carry-over (70). As the US is currently restricting the use of NT for infertility treatments, the patient was treated in Mexico. To date, one center in the UK is already applying NT technology to overcome the transmission of mtDNA diseases, and is extensively following up the health of the babies born (71, 72).

Nuclear Transfer for Female-Related Infertility Treatments

Nuclear Transfer to Treat Fertilization Failure and Embryo Developmental Arrest

Infertility affects 8% to 12% of couples worldwide, and both female and male factors may contribute to it (73). The evolution of ART has helped many couples worldwide to deliver a healthy baby, but the treatment of some couples remains a challenge. Two not well characterized cases of infertility are failed fertilization (FF) following ICSI and embryo developmental arrest (EDA) (74, 75).

Although ICSI has offered promising results in the field of ART with fertilization rates of 70% to 80%, FF still occurs in 1% to 5% of ICSI cycles (76). Oocyte activation deficiencies are the main reason for FF and can be attributed to both oocyte- and sperm-related factors. Following sperm injection, the sperm initiates a series of events in order to activate the arrested metaphase II oocyte. Following fertilization, a rise in Ca^{+2} peaks occurs within the oocyte, which is important for its activation and subsequent embryo development (77). In patients with FF after ICSI, these peaks can be abnormal or absent. Currently, assisted oocyte activation (AOA) has been proven beneficial for most of these patients. AOA involves the production of Ca^{+2} oscillations artificially by different methods, such as the use of calcium ionophores (78, 79). Albeit promising for FF related to sperm-related deficiencies (80), when it comes to oocyte-related factors, AOA is not always efficient in these patients (81), who have to seek for oocyte donation (82). Oocyte factors are attributed to compromised cytoplasmic quality, such as reduced mitochondrial numbers or abnormal proteins involved in fertilization (83, 84). Up to now, only mutations in four female genes (*PATL2*, *WEE2*, *TLE6* and *TUBB8*) have been linked to FF (85), while AOA was not beneficial for women with *WEE2* mutations (86, 87). Nevertheless, injection of the *WEE2* cRNA led to successful activation of the affected oocytes, allowing the formation of blastocysts (85), suggesting that cytoplasmic incompetence can be overcome by enriching the oocyte with the normal cRNA. The use of NT could possibly help these patients when a sperm-related factor is excluded. The compromised cytoplasm of the affected oocytes could be replaced by the cytoplasm of a donor oocyte by transferring the genetic material of the patient to the donor oocyte. There are currently no publications suggesting the use of NT to rescue FF in patients with oocyte activation deficiency factors, as oocyte factors are not yet well characterized. Oocyte factors are complex to study, not only because oocytes are scarce for research purposes, but also because a large number of maternal factors are involved in the oocyte activation process (88). Specifically, errors can occur in the oocyte Ca^{2+} realizing machinery, in the pathways activated downstream the Ca^{2+}, in the channels and pumps involved in Ca^{2+} homeostasis, but also due to a poor overall oocyte quality or nuclear defects (88).

Another not well understood condition is embryo developmental arrest. EDA is characterized by the primary arrest of embryos in the early cleavage stages (75). Approximately, 10 to 15% of IVF embryos arrest permanently, and some patients present recurrent complete embryo developmental arrest (89). Before embryonic genome activation (between four and eight cells in human), embryonic development is almost exclusively regulated by maternal RNAs and proteins stored in the cytoplasm (50). The genes encoding for these essential maternal factors are the so-called maternal effect genes (MEGs). Over 60 oocyte-specific MEGs have been found to be critical for mammalian development (90). However, research in human is still limited, and only few MEGs, some of which form the subcortical maternal complex (SCMC) have been identified. The SCMC is a multiprotein complex, composed of at least six proteins, that participates in the zygote genome activation, but its exact functions are still under debate (91). Recently, mutations in the genes involved in the formation of the SCMC, such as *TLE6*, *PADI6*, *NRLP2*, *NRLP5*, *NRLP7*, and *KHDC3L* have been detected in patients suffering from EDA (92–96). For patients facing EDA, the only current solution remains oocyte donation. As a treatment to embryo developmental arrest, NT could be proposed.

Two recent publications (42, 43) investigated the use of NT technology to overcome embryo developmental arrest in a mouse model. Nuclear transfer (both ST and PNT) between oocytes from NZB/OlaHsd mice that display a two-cell blockage and control B6D2 mice, rescued the embryonic development, resulting in high blastocyst rates (42). The use of ST by of Costas-Borges et al (43), using the same mouse model, demonstrated similar results but also low carry-over rates of maternal mitochondrial DNA and low heteroplasmy levels in the offspring for several generations, as well as normal fertility of the pups from the reconstructed embryos (43).

A recent study by Bai et al. (97), reported on the use of different NT techniques to overcome embryo developmental arrest in a *Zar1*−/− mouse model. *Zar1* is an important regulator of maternal genome degradation and embryonic genome activation. *Zar1*−/− mice displayed embryo developmental arrest. In order to rescue the development of these embryos, NT technology was applied, including ST or PB1T between *Zar1*−/− and wild type mouse oocytes, or early and late PNT between *Zar1*−/− and wild type zygotes. ST, early PNT, and PB1T significantly increased the blastocyst stage of the reconstructed oocytes/zygotes and also led to live offspring in

17.2% for early PNT, 32.6% in the ST group, and 29% for the PB1T group, comparable to the control group. Furthermore, the resulted offspring were healthy and fertile. Nevertheless, the delivery rate for late PNT was only 2.82% in the reconstructed zygotes (97).

In human, the first NT report to overcome embryonic arrest was reported in 2016 by Zhang et al. (98). PNT was applied for a patient with recurrent embryonic arrest at two-cell stage, following fertilization. The transfer of the pronuclei into the zygote of a donor, resulted in five four-cell stage embryos and a triplet pregnancy, although no live birth was achieved (98).

FF and EDA remain the challenges for ART, and cytoplasmic oocyte quality is of great importance for appropriate embryonic development. Current data are encouraging but not sufficient to support the use of NT for infertility treatment. Yet, two clinics in Greece and Ukraine are claiming live births by making use of the NT technology for female-related infertility, but peer-reviewed publications are currently lacking.

Nuclear Transfer for Advanced Maternal Age

In IVF clinics, women over the age of 37 years remain a challenging population for ART. Advanced maternal age is accompanied by ovarian aging, which is characterized by a decline in both quantity and quality of oocytes (99). Poor oocyte quality in aged women is associated with cytoplasmic deficiencies and impaired mitochondrial function, which has a negative impact on the ATP supply to support oocyte maturation and embryo development (100, 101). The mtDNA copies in a cell are directly correlated with its metabolic needs. In mature human oocytes, for instance, the number of mtDNA copies is approximately 100,000–600,000 (62). Importantly, a threshold of mtDNA copies has been suggested for successful fertilization and subsequent embryonic development (101). Women of advanced maternal age generate more aneuploid embryos compared with younger women (102). An oocyte has high energy demands for the formation of the meiotic spindle and the correct alignment of the chromosomes, but also to complete maturation, fertilization, and support the first cleavage stages of embryonic development (101). Mitochondria are maternally inherited, and no mitochondrial replication occurs before the blastocyst stage. Thus, the number of mtDNA copies in the oocyte is important for the first steps of embryonic development (100). The mtDNA copy number in the oocytes of older women is significantly decreased compared to those of younger women (103). This number is also reduced in the early cleaved embryos, while it is higher in blastocysts of older women. Nevertheless, this high number of mtDNA copies in blastocysts of older women has been associated with increased aneuploidy and failed implantation (104).

Despite the mitochondria, other cytoplasmic factors are also important for fertilization and proper embryonic development, such as organelles, metabolites, maternal RNAs and proteins, as descripted in the previous section (38, 105). A recent study by Bertoldo et al. (106), reported that poor oocyte quality from reproductive aged mice was associated with reduced levels of the metabolic cofactor nicotinamide adenine dinucleotide (NAD^+). Supplementation of the NAD^+ precursor Nicotinamide mononucleotide restored oocyte quality and enhanced blastocyst quality and live birth rates in the aged females (106).

Several methodologies have been explored to overcome the poor cytoplasmic quality of women with advanced maternal age. Cytoplasm transfer (CT), which involves injection with a limited portion of cytoplasm from a competent (donor oocyte) to an incompetent oocyte (107) and Autologous Germline Mitochondrial Energy Transfer (AUGMENT) have been investigated (108). The safety and benefit of CT remain unclear owing to certain abnormalities observed in the resultant children (109) although it is not certain that these abnormalities were caused by CT. Clinical applications of this technology were put into practice before extended animal studies, although a recent paper from Tang et al. demonstrated that CT was not beneficial to overcome cytoplasmic inferiority of the oocytes from old mice, in contrast to NT (42). Alternatively, the method of AUGMENT has been investigated. AUGMENT involves the supplementation of the incompetent oocyte with autologous mitochondria, isolated from oogonial stem cells harvested from an ovarian biopsy of the patient (108). Nevertheless, it is difficult to confirm the efficacy of the technique due to the small number of patients treated and also due to the difficulty of isolating oogonial stem cells and the controversy around their existence in the human adult ovary (110–113). Furthermore, a recent study reported no benefit in embryo quality in women with multiple IVF failures using the AUGMENT technology (111).

NT has been proposed for the indication of advanced maternal age. In 2009 Mitsui et al. (114), demonstrated the effectiveness of ST to rescue poor development in embryos originating from aged mice. Oocytes from young mice were used as recipients and high blastocyst rates were achieved (114). Tang et al. (42) achieved also promising results making use of ST and PNT. Furthermore, spindle assembly and mitochondrial potential were assessed in oocytes of mice of advanced age. A significantly higher number of abnormal spindles and misaligned chromosomes were noticed in the oocytes of aged and very aged mice compared with oocytes from young mice (42). Furthermore, mitochondrial membrane potential, representative of mitochondrial function, was severely compromised in the aged and very aged mouse group. Mitochondrial membrane potential values were increased in reconstructed oocytes with spindles from very aged mice transferred in the cytoplasm of young mice (42). Fertilization and blastocysts levels following sperm injections were significantly lower in the aged and very aged mouse group compared to the one of young mice. PNT significantly increased the fertilization and blastocyst rate of the reconstructed oocytes in both aged and very aged groups, after transfer of the pronuclei into enucleated zygotes from young oocytes. ST also increased fertilization and blastocyst formation for the reconstructed oocytes of the aged group but did not improve the results in the very aged mouse oocytes. Importantly, euploidy rate was very high in embryos originating from the reconstructed NT oocytes/zygotes. Opposite results were

observed with the transfer of the spindle or pronuclei from young mice in the cytoplasm of very aged mice (42). These results could indicate that ST and PNT might be able to avoid embryo aneuploidies created during embryo development, caused by the poor cytoplasmic quality of oocytes from mice of advanced maternal age. Nevertheless, the number of blastocysts analyzed in this paper was limited, and the results should be translated cautiously.

Use of Nuclear Transfer Technology for DOR/POR Patients

DOR patients are associated with poor reproductive outcomes, even when ART techniques are used (14). DOR patients usually exhibit POR due to compromised ovarian reserve (115). Poor oocyte numbers following stimulation regardless of the age of the patient or embryo quality have been associated with poor clinical results in this patient group (16). POR patients appear to be in a higher risk for foetal aneuploidies compared to normal response and higher chances for pregnancy loss, Down syndrome and other embryonic aneuploidies have been associated with patients with DOR (116–118).

In aged women with POR, poor pregnancy rates have been reported, as a normal sequence of ovarian ageing. As previously described, advanced maternal age is characterized by poor oocyte cytoplasm which highly compromises embryonic development and pregnancy rates (119). Despite the normal fertility decline associated with age, it is unclear whether this patient group is associated with higher embryonic aneuploidies compared with age matched control women. POR patients have similar fertilization, implantation, aneuploidy, and miscarriage rates compared to aged women with normal response to gonadotropins (16). Nevertheless, the number of embryos available significantly decreases in POR patients, affecting the chances for embryo transfer (16).

Poor oocyte and embryo quality do not seem to be the case for young women with POR (37, 39, 40). Young patients with POR seem to have similar fertilization rates and good embryo quality compared to age matched control women. Nevertheless, again, the number of oocytes retrieved following stimulation affects the number of available embryos for embryo transfer, resulting in decreased implantation and LBR. When a blastocyst is acquired, LBR is comparable to the women with normal ovarian reserve (39). One of the most important factors in the outcome of ART is the number of recruited oocytes following ovarian stimulation (120). A good yield of oocytes renders higher chances for a sufficient number of euploid embryos (121).

Since cytoplasmic quality and oocyte numbers are important factors for the outcome in the ART setting, NT could assist these patients. For the patients with advanced maternal age, DNA from the patient's oocytes could be transferred to the cytoplasm of a healthy young donor. In addition, the low number of oocyte yields from DOR/POR patients could be overcome by the use of NT. Here, we are proposing the use of three different NT techniques that would yield four embryos instead of one, starting from one patient oocyte (**Figure 2**).

The first step involves the transfer of the 1st polar body (PB1T) into an enucleated mature oocyte of a donor. Following fertilization with the sperm of the patient's partner, second polar body extrusion occurs with the formation of pronuclei, belonging to the patient's genomic DNA and the one of her partner's. The remaining spindle in the patient's oocyte can be transferred to a second enucleated donor oocyte and results in a zygote and second polar body extrusion following fertilization. Two more donor oocytes can be used for the reconstruction of embryos using the two second polar bodies after ST and PB1T. Donor oocytes are enucleated and fertilized with sperm from the partner of the patient. A single male pronucleus is formed. The second polar bodies from the reconstructed oocytes can be transferred to the zygote and results in two more embryos. The technique of this novel PB2T in human has been successfully optimized recently by Tang et al. in a research setting (48).

According to the above scheme, making use of only one patient oocyte, four embryos could be reconstructed with the help of NT technology. This would allow more available embryos to be chosen for embryo transfer in this patient group.

CHALLENGES IN NUCLEAR TRANSFER TECHNOLOGY

Despite the promising results of the NT technology in some animal and human models, results remain scarce on the value of this technique in clinical practice when mitochondrial diseases are not involved. Furthermore, a number of concerns have been raised over the years about the safety of the technique for the offspring.

Since mitochondrial function is under dual control of both nuclear and mtDNA, concerns are raised towards the possible incompatibility of the nuclear DNA (patient) and mtDNA (donor), which may occur from different mtDNA haplogroups. Diversity in the mtDNA sequence has been identified between individuals, with different haplotypes in mtDNA copies. Individuals fall in different haplogroups, depending on the characteristics of the mtDNA variants. These haplogroups were established during human evolution, and they are characteristic of some geographical regions (66). Nuclear–mitochondrial incompatibility has been previously reported in an animal study, reporting high embryonic lethality and stillborn rates in mice, when applying nuclear transfer between two mouse breeds (122). Nevertheless, health reports in other studies suggest that nuclear–mitochondrial incompatibility is not an issue (68, 70). Furthermore, current evidence also suggests that nuclear transfer does not affect the mitochondrial function in humans. This was shown in Embryonic stem cells (ESCs) from reconstructed blastocysts using NT. ESCs had similar mitochondrial respiratory chain enzyme activity and oxygen consumption rates regardless the combination of nuclear–mitochondrial DNA (53, 123).

Another reason for caution when applying NT is the occurrence of cytoplasmic carry-over of mutant mtDNA molecules from the patient's to the donor oocyte. Even though reported levels of mtDNA carry-over in reconstructed NT

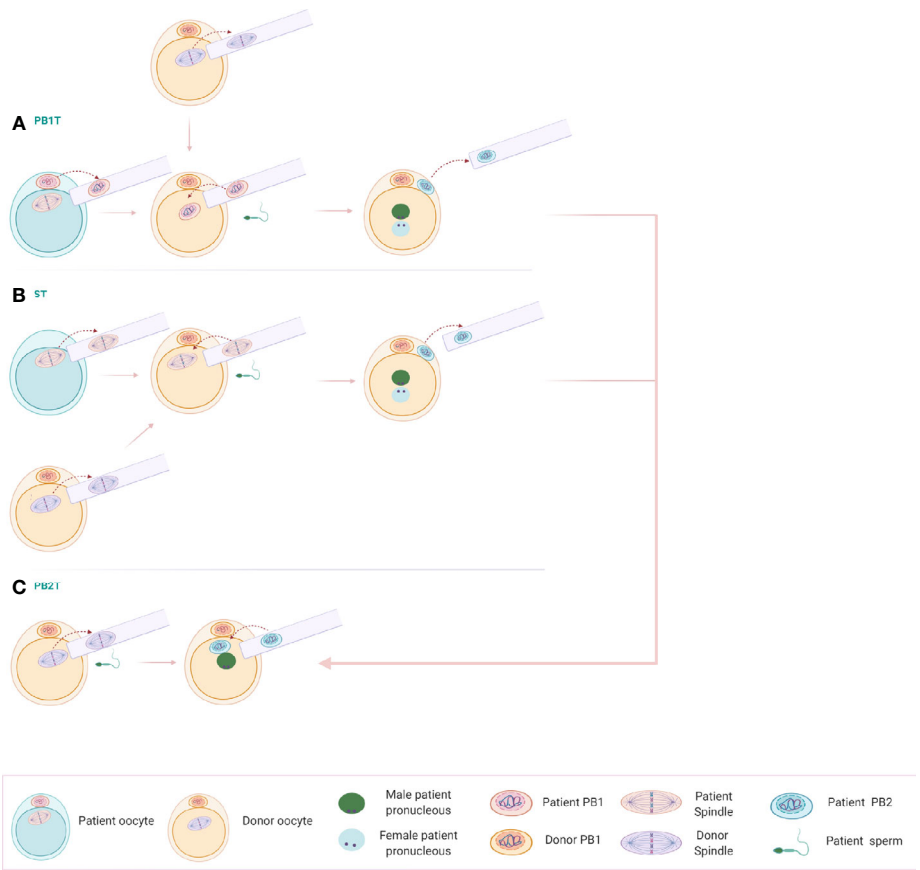

FIGURE 2 | **(A)** Polar body 1 transfer (PB1T): The first polar body of a mature oocyte is transferred to a donor mature oocyte from which the spindle has been removed. Following reconstruction, the oocyte is fertilized, extruding the second polar body. **(B)** Spindle transfer (ST): The spindle of the patient's oocyte can be transferred into an enucleated donor metaphase II (MII) oocyte. The reconstructed oocyte can be fertilized with the patient's sperm and extrude the second polar body. **(C)** Polar body 2 transfer (PB2T): An oocyte of the donor is enucleated and fertilized with the sperm of the patient's partner. A single pronucleus is being formed, containing only the genetic material of the partner. Polar body 2 resulting from PB1T or ST can be transferred to the zygote, including now the genetic material of the patient and the correct genetic load.

embryos were always lower than the 18% threshold level for disease expression (51, 52), the occurrence of heteroplasmy drift could cause a shift in heteroplasmy levels during development. A recent study demonstrated a competition between different mtDNA haplotypes. The heteroplasmic mouse model containing the C57BL/6JOlaHsd nuclear genome and either NZB/OlaHsd or C57BL/6JOlaHsd mtDNA showed that one of the mtDNA haplotypes was becoming predominant, termed as "haplotype selection", during oogenesis and early embryo development, which was dependent on the specific interaction between the nuclear and mitochondria encoded genes (61). Despite the low heteroplasmy levels in reconstructed human embryos, progressive increase in the mtDNA heteroplasmy levels of several hESC lines derived from reconstructed NT blastocysts has been reported (52, 123). Whether this is due to the artificial nature of hESCs or a biological phenomenon has to be further explored. Since the effect of heteroplasmy on the reconstructed embryo has not been elucidated yet, the minimal carry-over should be guaranteed. The most studied nuclear transfer technologies are ST and PNT. Several studies have reported different levels of carry-over to the reconstructed embryos by the application of the two techniques; nevertheless it seems that these two techniques allow a very small amount of mtDNA to be transferred from the nuclear donor. The minimum carry over seems to occur with the use of PBT, as polar bodies have a very small cytoplasmic content (47, 62).

Another limitation for the use of NT technology is that it would increase the financial cost for the patients. This should be avoided until the benefits and the safety of the technique are more concrete for patients with female subfertility. Nevertheless, these techniques are not so labor intensive, as especially the model we are proposing can be done during the daily IVF routine, and PB2T can occur early the next morning. Yet, a microscope for spindle visualization is required and due to the sensitivity of the material, users should be well-trained. It is worthy to note that NT cannot correct all aspects of infertility. If

genetic anomalies already exist in the spindle or polar bodies of the mother, something that is highly prevalent in aged women (124), then the NT will not be of benefit (42). In this regard, PGT to assess aneuploidy should be offered in all cases when NT embryos are being created as the NT technique is still very novel.

Before any further application of this technology, the ethical aspects should be considered thoroughly.

The ethical concerns primarily refer to the genetic modification of the germline. Some argue that NT could cause genetic modifications that could be transmitted to the next generation. Nevertheless, unlike other technologies, NT does not target the DNA of the genome nor of the mitochondria (125). Although strict regulations and federal organizations have been established in some countries that control the creation of embryos for research purposes, prohibiting any use for eugenic intent, the use of human embryos remains a debatable issue (126). Furthermore, a number of people argue that women donating oocytes for research could be exploited, and the donated oocytes could be seen as a commodity to experiment the different research techniques (127). The genetic contribution of the donors has also been criticized. Nevertheless, in the case of mitochondrial donation, nuclear DNA from the donor is not contributing to the offspring, in contrast to other cases of oocyte donation, only the mitochondrial DNA. Finally, one of the most important ethical concerns for the use of NT is the safety of this technique to the offspring (127). Although available studies are promising, the number of applications in human is limited, and therefore, the technique should be considered as highly experimental, and thorough follow-up of the children born after this technology should occur.

CONCLUSIONS

DOR patients remain a challenge for current ART practice. Poor response to gonadotropins and poor oocyte quality lead to the recruitment of a lower number of good quality oocytes for fertilization. NT is a new technology being used to overcome the transmission of severe mitochondrial diseases from mother to offspring (128). Lately, this technology has been proposed for certain types of female-related subfertility (98), but scientific reports remain scarce. Making use of the proposed NT scheme described above, we believe that a higher number of embryos can be reconstituted for DOR patients when making use of these various NT strategies. This approach is expected to also overcome cytoplasmic defects in oocytes of women of advanced age. Before any application of NT for DOR patients, more studies should be carried out in animal models before assessing the safety of the technique on patients suffering from subfertility and PGT should remain the tool to ensure the safety of the reconstructed embryos.

AUTHOR CONTRIBUTIONS

AC and BH designed the idea of the review. AC and BH wrote the manuscript. AB, MT, CD, and DS provided scientific input, corrected, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Stem Cell Paracrine Signaling for Treatment of Premature Ovarian Insufficiency

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Premature ovarian insufficiency is a common disorder affecting young women and represents the worst-case ovarian scenario due to the substantial impact on the reproductive lifespan of these patients. Due to the complexity of this condition, which is not fully understood, non-effective treatments have yet been established for these patients. Different experimental approaches are being explored and strategies based on stem cells deserve special attention. The regenerative and immunomodulatory properties of stem cells have been successfully tested in different tissues, including ovary. Numerous works point out to the efficacy of stem cells in POI treatment, and a wide range of clinical trials have been developed in order to prove safety and effectiveness of stem cells therapy—in diminished ovarian reserve and POI women. The main purpose of this review is to describe the state of the art of the treatment of POI involving stem cells, especially those that use mobilization of stem cells or paracrine signaling.

Keywords: follicular rescue, ovarian rejuvenation, premature ovarian insufficiency, stem cells, autologous stem cell ovarian transplant, mobilization

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INTRODUCTION

In humans, oocyte development begins during fetal life and follicle pool reaches its maximum at 16/20 weeks of fetal development (1). Follicular decline is initiated before birth, so that, at the time of delivery, only about 1 million of follicles remain in the ovary of the baby. By the time of menarche, each ovary contains about 400,000 follicles and ovarian reserve continues decreasing as women age (2). Thereby, the decline in oocyte quantity and quality during women reproductive life is a physiological process; however, in some women, ovary deterioration occurs in an abrupt way and they become prematurely infertile.

Primary Ovarian Insufficiency (POI), also known as Premature Ovarian Failure (POF) or premature menopause, is a reproductive disorder, characterized by oligo-amenorrhea and high levels of serum FSH, leading to a cessation of ovarian function before the age of 40 (3). This condition affects 1% of women under the age of 40 years, and 1 out of 250 women under the age of 35 years (4).

POI is characterized by a hypergonadotropic hypogonadism state, which can be diagnosed by a triad of features in a woman under the age of 40: (a) postmenopausal levels of follicle-stimulating hormone (FSH) (>40 UI/L in two different samples taken separately in the time), (b) 4 or more

months of amenorrhea, and (c) decreased estradiol serum concentrations (3). These patients present low AMH serum levels and a low antral follicle count (AFC).

This condition represents a dramatic scenario, as ovarian dysfunction leads to female infertility in POI patients. Laparoscopy shows a lack of follicle development in POI patients and dysfunctional ovaries lead to estrogen deficiency. The uterus and vaginal mucosa undergo atrophy, which is very often associated with dyspareunia (5, 6). In addition, POI involves menopausal syndrome, which may include hot flushes, night sweats, heart palpitations, insomnia, or headaches. Moreover, POI is associated with long-term negative consequences in female health, such as an increased risk of immunological disorders, cardiovascular diseases, and osteoporosis (7).

It should be noted that 5–10% of women with POI might have spontaneous follicular development, menses resumption, or spontaneous pregnancies, especially during the first year after diagnosis (8). This could be due to the fact that ovarian biopsies from POI patients revealed that up to 9% of women have plenty primordial follicles, and 30% have some primordial follicles (5, 9). However, ovulation is unpredictable and most women with POI have a low chance of pregnancy (5, 6, 10).

There are limited options for POI patients, whose treatment may be oriented to reduce the impact of endocrine dysfunction—by means of therapy hormone replacement—and/or to overcome infertility. None of these strategies are absent from limitations. On the one hand, hormone replacement therapy has been associated with an increased risk of reproductive cancer (11–15). On the other hand, the treatment of POI-associated infertility by reproductive techniques with autologous gametes represents a major challenge in reproductive medicine, and usually involves prolonged protocols and inconsistent clinical outcomes. Despite the intensive effort to develop variants of stimulation protocols to improve reproductive outcomes in poor responder patients, intrinsic characteristics of POI patients make its management even more difficult; probably, because of the absence of antral follicles responding to stimulation. To date, there is no enough evidence to recommend most of these strategies in order to improve pregnancy rates in POF patients (16, 17). In most cases, the only options for these patients to achieve desired motherhood is oocyte donation or adoption, which are not always accepted due to ethical, cultural, or religious issues. Thus, because of the complexity of this disorder, a standard and effective treatment has not yet been established; but an active management of these patients and an intensive search for new strategies may open new doors for them.

ETIOLOGY

The human ovary contains a limited and non-renewable pool of quiescent follicles determined at birth. Folliculogenesis is a complex process that should be extremely regulated. During this process, granulose and theca cells assist the oocyte, in order to promote primordial follicle development towards antral stage and ovulation (10) (**Figure 1**). Intraovarian mechanisms activate a small number of primordial follicles (≈1,000/month) and although most underwent atresia, a few of them achieved the advanced maturation stage before ovulation (18). Follicle depletion occurs at menopause when less than 1,000 quiescent follicles remain (19). In POI, this process is altered. It is suggested that follicular dysfunction and altered follicle depletion may underlie POI (20). Although scientific knowledge is limited about factors controlling oocyte pool and the cause of POI is not yet completely understood, different factors could alter follicle maintenance and development. In fact, POI can appear spontaneously or induced by different factors (21).

The most common cause of POI are oncologic treatments with high doses of chemo- and radiotherapy (22). The increased survival rates (>80%) of oncologic patients associate a growing percentage of young women facing gonadotoxic side effects of cancer therapies without having accomplished their reproductive

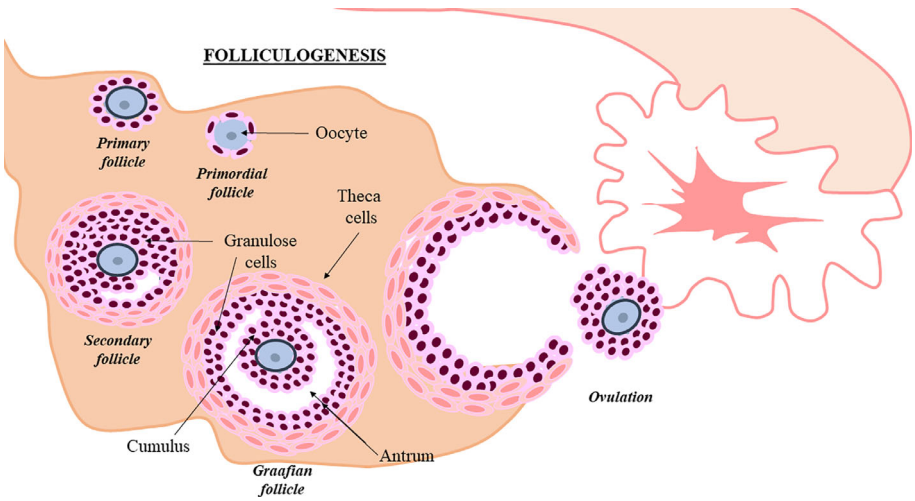

FIGURE 1 | Follicullogenis. Granulosa and theca cells assist oocyte progression towards ovulation (10). Follicle development is shown in the picture.

project. Deleterious effects on the ovary depend on the age of the patients—the risk to develop POI after cancer therapy increases with the age—the dosage and type of toxic agent (having the alkylating agents the highest risk for developing POI). A main mechanism of chemotherapy-induced ovarian failure is based on the damage induced to DNA of primordial follicles, leading to apoptosis, and promoting a massive activation of follicles followed by atresia and elimination. This follicular depletion also associates an impairment of ovarian vascularization, fibrosis, or interrupting cross talk communication between follicular cells (23, 24), leading to a cessation of ovarian function

Iatrogenic factors, such as laparoscopy, ovarian drilling, or surgery for ovarian endometriosis or cysts, may also lead to POI (25). Others environmental factors such as viral infections or pollutants can result in POI, although the real incidence of these cases is not clear (26, 27).

Genetic defects, including X chromosome aneuploidies (Turner syndrome, trisomy X) (28–30), structural X chromosome anomalies (isochromosome, deletions, inversions, duplications) (31), mutations or premutations of X linked genes (Fragile X syndrome) (31), and single mutations in genes related to reproductive function (FSH receptor, LH receptor, inhibin, galactosaemia) constitute another cause of POI (32). Enzymatic deficiencies in the steroidogenesis pathway could also lead to POI (22).

Finally, autoimmune mechanisms are involved in pathogenesis of more than a 4% of POI cases (33) and autoimmune disorders such as myasthenia gravis, celiac disease, vitiligo, lupus, Addison's disease, or autoimmune polyglandular syndrome, have been seen in a percentage of women diagnosed with POI (22, 34). In these patients, immune alterations including an increase in CD4+ T cells and B cells, macrophage and dendrite cells disorders, lymphocytes oophoritis, and inappropriate expression of class II MHC antigens by granulose cells have been found (35–37). In fact, anti-ovary antibodies—with several targets—have been detected in 50% of unexplained infertile patients and several studies report that the presence of autoantibodies increases the risk to develop POI in patients with autoimmune disease (22, 34).

However, most cases of POI are idiopathic (22), which promote further investigations to utterly understand this entity, in order to explore new strategies to solve it. Even in cases with a diagnosed cause, the diversity of disorders associated with POF indicates the heterogeneity of this entity. This fact underlines the need not only to develop different strategies to improve clinical management of these patients, but also the importance of the selection of the right population of POI patients, who can benefit from each approach.

NOVEL STRATEGIES FOR POI MANAGEMENT

Recent research has focused the attention on the residual quiescent pool of follicles that remain even when the ovary loses their ability to ovulate and function. Based on the successful protocol of *in vitro* activation (IVA) of primordial dormant follicles, Kamawura et al. was the first group exploring the combination of IVA with mechanical ovarian fragmentation, to inhibit the Hippo pathway, in menopausal women (38). Additional research has been developed to improve the success of the technique and to design a less invasive procedure, named as one step IVA or ovarian fragmentation for follicular activation (OFFA). The technique consists in a unique surgery for ovarian cortex retrieval, followed by mechanical fragmentation into small pieces and transplant into an ovarian grafting site. By means of this strategy, 10 premenopausal women have achieved a pregnancy (39–43) as well as several poor ovarian responders (POR) (44). OFFA pursues not only fertility recovery but also endocrine function and as avoids ovarian cryopreservation, the main concern of ischemia-associated follicle death is also overcome.

Artificial ovary is also a promising alternative that will be used to *in vitro* growth and maturation approaches or to improve ovarian transplant in the future. Although it has been applied successfully in animal models, its efficacy and safety have to be proved before it becomes a reality for patients (45).

Another strategy, closely related to the previous one, is the generation of artificial gametes in patients who are not able to produce functional gametes. Artificial gametes could be generated from induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs) derived from blastocysts inner cell mass, or the putative germline stem cells (GSCs) (45). In animals, artificial gametes have been generated from GSCs (46), ESCs (47), and iPSCs (48, 49). The implementation of this technique in animal preclinical models has even achieved the birth of viable offspring (48, 49). The main limitation of the technique—apart from multiple ethical concerns—is the low efficacy of differentiation. In humans, artificial oocyte-like cells have been developed from ESCs (50) and GSCs (46) and the successful fertilization of artificial oocyte-like cells has been reported (51). However, development potential and human offspring from artificial gametes is to date far from reality (52). In relation to this strategy, transplantation of ovarian granulosa-like cells derived from human IPSCs has been reported to repair ovarian niche and to promote follicular development in POF mice (53).

Different studies have described a lower telomere length and telomerase activity in POF patients (54–56). Concerning this pathway, the reactivation of telomerase—which maintains telomere length—has been described to resume fertility in telomerase-deficient mice, which present impaired fertility (57, 58), opening a new future possibility to ovarian rejuvenation by means of telomerase reactivation (59–61).

Based on the autoimmune dysregulation associated to a wide percentage of POI cases, together with the close association of fibrosis with ovarian failure, some authors suggested the possible effect of immunomodulating therapy for ovarian function recovery in POF patients, especially in those with autoimmune-related POF (34, 62, 63). In fact, use of the anti-inflammatory and antioxidant properties of several agents has been proved to improve ovarian function in a POI mice model (62, 64).

STEM CELL–BASED APPROACHES

Preclinical Studies

Most of these strategies highlighted the relevance of the ovarian niche as a key parameter to promote an adequate follicle development in order to restore ovarian function. Following this idea, one of the most promising strategies pursues the regeneration of ovarian niche using Stem Cells (SCs) in order to promote development of remaining follicles within the ovary.

With the rise of regenerative medicine, different types of SCs have been tested for follicular rescue and regeneration of the ovarian niche. Among them, **mesenchymal stem cells (MSCs)** have been the most widely used for these strategies. MSCs are a population of SCs that can be derived from different adult tissues, and that have proliferative, self-renewal and differentiation to different lineages properties (65). Different studies in animal models with different degrees of ovarian damage describe the ability of MSCs to restore ovarian function in these animals.

Mesenchymal stem cells from human and murine amniotic fluid have shown the ability to survive and proliferate in the ovary and to rescue short-term fertility of mice with chemotherapy (QT)-induced POF after injection into the ovarian artery (66, 67). Wang et al. report the ability of amniotic epithelial stem cells (AESCs) to infiltrate the damaged ovary after injection into the tail of mice with POF (68), leading to the recovery of folliculogenesis and differentiation towards granulosa cells (68, 69). Ding et al. show the recovery of the follicle pool in all its developmental stages and hormonal restoration after the tail injection of both AESCs and amniotic mesenchymal stem cells (AMSCs) in mice with different degrees of ovarian failure induced by chemotherapy (70). Although AESCs seem to show less immunological rejection, AMSCs show a higher efficacy in the recovery of ovarian function, especially in the most drastic cases of POF (70). Ling et al. evaluate the improvement of the treatment with human AMSCs by pretreating them with low intensity pulsed ultrasound (LIPUS). Both LIPUS-pretreated AMSCs and non-pretreated AMSCs have been reported to increase reproductive organ weights, reduce granulosa cells (GCs) apoptosis and ovarian inflammation, and improve ovarian function in POI rats (71).

MSCs can also be obtained from the umbilical cord. The injection of **umbilical cord mesenchymal stem cells (UCMSCs)** into the tail vein allows improvement of the ovarian structure and ovarian function—at the hormonal and follicular level—in mice with POF induced by QT and in rats with natural ovarian aging. GCs apoptosis reduction and cytokines secretion leading by UCMSCs are proposed as possible mechanisms of action (72–75). Zhu et al. report that the recovery of ovarian function and fertility after UCMSCs transplantation occurs sooner when UCMSCs are injected directly into the ovarian artery (76). It has also been reported a long-term survival of UCMSCs in the rat ovary after the transplant (73) and stabilization of the ovarian epithelium by these SCs (74). Both human UCMSCs and AMSCs interventions restore ovarian morphology elasticity and toughness, and involve a slight recovery of ovarian function in QT damaged ovaries (77).

Menstrual blood is another possible source of MSCs. **Human menstrual blood-derived stem cells (MenSCs)** are endometrial MSCs, which have also been used for the treatment of POI. Thus, the ability of these cells to migrate to the ovary has been reported, and ovary infiltration by MenSCs is followed by hormone levels restoration and follicular count increase in mice with QT-induced POF (78–80), as well as the restoration of fertility in these mice (78). The reduction of both fibrosis and apoptosis through cytokine secretion has been suggested as possible mechanisms to restore ovarian function by MenSCs (78, 79). Feng et al. also described a possible role of these cells in the regulation of folliculogenesis (80). Recovery of ovarian function has been also achieved by injecting only the culture medium of MenSCs, which reinforces the idea of paracrine action of these cells (79). Thus, MesSCs would represent an interesting alternative due to the possibility of non-invasive collection. Nevertheless, most of POI patients present amenorrhea or oligomenorrhea, which reduces the application of this strategy.

Adipose tissue-derived mesenchymal stem cells (ADMSCs) have also shown the ability to restore ovarian function, increasing the number of follicles after injection into the ovary in QT induced POF mice and rats (81–83). An improvement in estradiol serum levels and an increase in the gestation rate have been reported after ADMSCs transplant (82, 83). Fouad et al. report a therapeutic efficacy of both human AMSCs and ADSCs, but with a greater efficacy of the former, which achieve not only an increase in estrogen levels, but also a decrease in FSH levels in mice with POF (83). ADMSCs have been suggested to produce cytokines and reduce apoptosis in GCs (81). However, a low long-term permanence of these cells in the ovary has been reported (81). The transplantation of soluble collagen with ADSCs improves the short-term permanence of ADSCs in the ovaries and contributes to the restoration of ovarian function (82). Takehara et al. also describe a restoration of ovarian function after local injection of male ADMSCs into female mice with QT induced POF, and note that the Y chromosome only appears in theca cells and not inside the follicle. They report an increase in secreted levels of vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and hepatocyte growth factor (HGF) (84).

Although promising results obtained for ovarian rescue with different types of MSC, their clinical application requires cell culture procedures to reach clinically relevant cell numbers for transplant. This represents a main limitation for use, as the accumulation of genomic and epigenomic alterations and degeneration in progenitor potency in human SCs have been associated to cell expansion procedures (85).

Bone marrow derived stem cells (BMDSCs) present an interesting alternative for transplantation in women with POI (**Table 1**). The possibility of obtaining a large number of BMDSCs, from an autologous source, by means of well-established clinical protocols—used for BM transplant after QT—makes them a valuable candidate (96). This possibility lets us avoid cell expansion steps, which is associated with genetic instability (97). In 2007, Lee et al. described fertility rescue in a QT-induced POF rat model after injection into the

TABLE 1 | Animal studies involving bone marrow stem cell in POI/POF models.

Regenerative factor	Study population	Administration method	Main findings	Reference
Murine BMDSC	QT induced POF mice	IV tail injection	Fertility rescue. All the offspring come genetically from the recipient, but oocytes from the donor are described.	Lee et al. (86)
Murine BMDSC	QT induced POF rats	Direct injection in ovary	Ovarian function improvement. GCs apoptosis decrease.	Fu et al. (87)
Murine BMDSC	Natural aged mice	IV tail injection	Prolongation of reproductive potential	Selesniemi et al. (88)
Murine BMDSC	QT induced POF rats	IV tail injection	Ability of BMDSC to infiltrate damaged rat ovaries. E2 and AFC increase.	Liu et al. (89)
Murine BMDSC	FSH knockout mice	IV tail injection	Follicle count and mature follicles increased. Hormonal levels restoration. Size of reproductive organs increase.	Ghadami et al. (90)
Rabbit BMDSC	QT-induced POF Rabbit	IV ear injection	E2 levels and follicle count increase and FHS levels decrease.	Abd-Allah et al. (91)
Murine BMDSC	QT-induced POF mice	IV tail injection	Hormonal levels rescue. Healthy follicles increase and apoptosis decrease.	Bao et al. (92)
Murine BMDSC	QT-induced POF mice	Direct ovarian infusion	Fertility restoration and shortening of estrous cycles.	El Andaloussi et al. (93)
Human BMDSC	QT-induced POF mice	Direct ovarian infusion	Follicle count and ovarian weight increase. Hormone restoration and pregnancy rates improvement.	Mohamed et al. (94)
Human BMDSC	QT-induced POR and POR mice	IV tail injection	Preovulatory follicle and MII oocytes increase. Fertility restoration, pregnancy rates and litter size increase. Apoptosis reduction, vascularization, and cellular proliferation increase. Ability of BMDCSs to migrate and infiltrate xenotransplanted human ovaries, promoting vascularization, follicle development and E2 secretion.	Herraiz et al. (95)

BMDSC, Bone marrow derived stem cells; POI, premature ovarian insufficiency; POF, premature ovarian failure; POR, poor ovarian responder; AFC, antral follicle count; AMH, Anti-Mullerian hormone; IVF, in vitro fertilization; IV, intravenous; E2, Estradiol; TNF-α, Tumor Necrosis Factor α; IGF-I, Insulin-Like Growth Factor 1; GCs, Granulose Cells.

tail of BMDSCs derived from another rat. All the offspring belonged genetically to the recipient rat, although donor immature oocytes were described (86). Fu el al., in 2008 describe the improvement of ovarian function after the local injection in the ovary of BMDSCs derived from human in mouse with POF, possibly mediated by the reduction of apoptosis in GCs (87). The ovarian function improvement with BMDSCs has also been proved by prolongation of reproductive potential in mice beyond the common age of reproductive senescence with monthly infusions of BMDSCs from young mice (88). The increase in the number of follicles and the size of the reproductive organs, as well as the restoration of hormonal levels have been described in genetically generated POF mice, and QT induced POF mice, rats, and rabbits after heterologous distal BMDSCs transplantation (90, 91, 98). In 2014, Liu et al. describe for the first time the ability of BMDSCs to migrate into POF-damaged rat's ovary, where they were not distributed in a uniform manner. Their presence was described to be higher in the medulla than in the cortex. Following BMDSCs transplantation, increased estradiol levels and antral follicle count were reported (89). Different studies describe not only the regenerative potential of BMDSCs, but also a protective property, with a reduction of apoptosis in ovarian cells and lower germ cell DNA damage when combining chemotherapy with injection of BMDSCs (99, 100). Heterologous transplant of BMDSCs also shows the potential to reverse the hormonal dysfunction caused by QT in mice and an increase in the number of healthy follicles has been showed after BMDSCs tail vein injection (92). The ability of autologous BMDSCs transplant to restore fertility and shorten estrous cycles has also been reported after SCs injection in the ovarian artery in a QT-induced POF mouse model (93). After that, the ability of human BMDSCs transplant to increase ovarian weight and follicular count, and to improve pregnancy rate after injection into the ovarian artery has been shown in a mouse model with QT-induced POF (94). Herraiz et al. described the restoration of fertility in a mouse model with ovarian damage after transplantation of human BMDSCs, with an increase in the number of preovulatory follicles, MII oocytes, spontaneous pregnancy rate, and number of healthy offspring (95). Furthermore, this study shows for the first time, the ability of BMDSCs to migrate towards the follicles and vessels in human tissue POR women xenografted into immunodeficient mice, promoting follicular development, ovarian local vascularization, estradiol secretion, and reducing apoptosis (95).

In spite of the greater potential of MSCs, others SCs sources have been explored. Liu et al., described the potential of **human embryonic stem cell (ESCs)** to restore hormone levels and increase follicular count in mice, after the vein injection in vesicles, possibly by means of the apoptosis reduction (101). However, the use of ESCs, which may be obtained from the blastocysts inner cell mass is not exempt from ethical concerns.

In light of reported studies and advances in this field by using preclinical animal models, there are higher expectations regarding the use of MSCs, and especially BMDSCs to restore ovarian function in humans.

Pilot Studies and Clinical Trials in POI Patients

The firsts clinical trials developed in humans using MSCs from bone marrow (BM) required iliac crest aspiration for cell collection followed by SCs isolation and *in vitro* culture procedures to reach clinically relevant cell numbers (**Tables 2.1** and **2.2**).

Gupta et al. (17) published a live birth in a postmenopausal woman (45 years old) underwent this technique and IVF treatment. They injected cells in both ovaries by laparoscopy. This means to expose the patient to two different invasive procedures: first the iliac crest aspiration, and second the laparoscopy. Edessy's group followed the same technique, in 10 POI younger women (26–33 years old) with positive results showing a return of menses in two patients and one ongoing pregnancy, with one live birth (104). Gabr et al. (105) later applied this same method in 30 POI women (18–40 years old).

TABLE 2.1 | Human studies involving bone marrow stem cell treatment for POR patients.

Regenerative factor	Study population	Administration method	Main findings	Limitations	Reference
BM-MSC	33 patients with idiopathic/other POF/ POI and low ovarian reserves. Baseline characteristics not yet reported.	BM-MSCs into both ovaries *via* laparoscopy.	Not yet reported	Still ongoing	Al-Hendy et al. (NCT02696889)
BMDSC	17 POR patients (<40 years old). AMH = 1.9 ± 0.6 pM AFC = 4.0 ± 1.3	One ovarian artery by intraarterial catheterism	-81.3% POR improved AFC and AMH 2 weeks after treatment. - 33.3% treatment PR. - 5 pregnancies and 3 live births.	16% euploidy rate due to advanced maternal age was not ameliorated.	Herraiz et al. (102)

BM-MSC, bone marrow mesenchymal stem cells; BMDSC, bone marrow derived stem cells; POI, premature ovarian insufficiency; POF, premature ovarian failure; POR, poor ovarian responder; AFC, antral follicle count; AMH, anti-Mullerian hormone; FSH, follicle stimulating hormone; COS, controlled ovarian stimulation; IVF, in vitro fertilization; GSC-F, granulocyte colony-stimulating factor; ASCOT, autologous stem cell ovarian transplantation. Modified from Herraiz et al. (103).

TABLE 2.2 | Human studies involving bone marrow stem cell treatment for POI and perimenopausal patients.

Regenerative factor	Study population	Administration method	Main findings	Limitations	Reference
BM-MSC	1 perimenopausal woman (45-year old). AMH 0.4 ng/ml AFC = 1	BM-MSCs into both ovaries *via* laparoscopy.	-AFC and AMH increased 8 weeks after treatment. -1 live birth.	POR similar to that reported for POI patients without treatment	Gupta et al. (17)
BM-MSC	10 women with idiopathic POI (26–33 years old). AMH <0.1 ng/ml; FSH = 58 mIU/ml	BM-MSCs into both ovaries *via* laparoscopy.	-Resumption of menses in 20% patients after 3 months. -10% treatment POR. -One pregnancy and a live birth in one patient showing endometrial regeneration.	POR similar to that reported for POI patients without treatment	Edessy et al. (104)
BM-MSC	30 patients with POF (18–40 years old). Baseline characteristics not reported.	Direct laparoscopic infusion into the ovarian stroma and catheterism into the ovarian artery of one side.	-86.7% POF patients improved hormone profile 4 weeks after treatment. -60% showed ovulation. -3 patients underwent IVF. -1 spontaneous pregnancy.	-AFC not reported or compared between ovaries. -IVF outcomes were not reported.	Gabr et al. (105)
BM-MSC	33 patients with idiopathic/other POF/ POI and low ovarian reserves. Baseline characteristics not yet reported.	BM-MSCs into both ovaries *via* laparoscopy.	Not yet reported	Still ongoing	Al-Hendy et al. (NCT02696889)
BMDSC	20 POI patients (<39 years old). (10 patients included) Baseline characteristics not reported	One ovarian artery by intraarterial catheterism (ASCOT) (6 patients) and stem cells mobilization to peripheral blood by means of GSC-F (4 patients).	-Follicular development in both arms (90–140 days after treatments). -AFC increase in 50% of patients (GSC-F arm) and 66.6% of women (ASCOT arm). -Statistically significant FSH decrease is not observed, although FSH decreased was decreased. -In G-CSF arm: COS initiated in 2/4 women and 1 embryo vitrified. Embryo transfer was performed but pregnancy was not achieved -In the ASCOT arm: COS initiated in 4/6 women, 1 embryo vitrified and transferred, having an ongoing pregnancy. -Menses recovery in 40% of patients and climacteric symptoms decrease in 50% of women.	Still ongoing. Preliminary data from interim analysis reported (106)	Herraiz et al., (NCT03535480)

BM-MSC, bone marrow mesenchymal stem cells; BMDSC, bone marrow derived stem cells; POI, premature ovarian insufficiency; POF, premature ovarian failure; POR, poor ovarian responder; AFC, antral follicle count; AMH, anti-Mullerian hormone; FSH, follicle stimulating hormone; COS, controlled ovarian stimulation; IVF, in vitro fertilization; GSC-F, granulocyte colony-stimulating factor; ASCOT, autologous stem cell ovarian transplantation. Modified from Herraiz et al. (106).

This study, instead, had two branches: one arm received these cells by direct ovarian injection through laparoscopy, while the second arm had cells injected through de ovarian artery. One spontaneous pregnancy was obtained. Al Hendy and colleagues are carrying out similar studies as the above described in POR and POI patients, after their promising results in animals, but their investigations are still ongoing.

We recently described that infusion of BMDSC promotes human and mouse follicular growth by increasing ovarian vascularization, stromal cell proliferation, and reducing cell death (95). Based on this information, a prospective pilot study in 17 POR women was developed by our group to evaluate the effects of autologous stem cell ovarian transplant (ASCOT) on ovarian reserve (102). ASCOT improved ovarian function biomarkers (AMH and AFC) in 81.3% of women and a total of six pregnancies and three healthy babies were achieved. ASCOT improved follicle and oocyte quantity enabling pregnancy in POR women previously limited to oocyte donation. In the context of ovarian tissue, stem cell paracrine actions should be evaluated for their capacity to activate the pre-existing quiescent follicles based on the ability of BMDSCs to produce and secrete a broad variety of growth factors involved in follicular growth, angiogenesis, viability, and ovarian response to Controlled Ovarian Stimulation (COS) (107). In fact, our results suggest that ASCOT optimized the growth of existing follicles, mediated the presence of specific stem cell secreted factors such as FGF-2 and THSP-1 within aphaeresis. Based on that, a randomized pilot study (NCT03535480) has been designed with 20 POI women younger than 39 (106). Patients will be randomized to the ASCOT or only stem cell mobilization based on the ability of ovarian niche to attract undifferentiated cells from BM in a process known as "homing" (89).

It is important to highlight that many other stem cell origins are also being tested worldwide in several RCT involving POI women. Nevertheless, most of them are still ongoing and therefore results have not been yet reported (**Table 3**).

TABLE 3 | Registered Randomized Clinical Trials involving different sources of SCs—apart from Bone Marrow Derived Stem Cells—for POF/POI patients.

Regenerative factor	Study population	Inclusion criteria	Number of clinical trial	Status
Human umbilical cord mesenchymal stem cells (hucMSCs)	12 patients with POF	-Diagnostic criteria of ESRHE -No hormonotherapy within 3 months	NCT03816852	Suspended
Human umbilical cord mesenchymal stem cells (hUCMSCs) and human cord blood mononuclear cells (hCBMNCs)	40 patients with POF	-Age: 18–39 -Clinical diagnosis of POF -Currently receiving Hormone Replacement Therapy	NCT01742533	Unknown
Human Embryonic Stem Cell Derived Mesenchymal Stem Cell (MSC)-Like Cells	18 patients with POF	-Age >40 -Have established regular menstrual cycle, oligomenorrhea/amenorrhea ≥4 months -FSH >25 IU/ml -Bilateral ovaries visible by ultrasound -Fertility requirement and sperms in couple	NCT03877471	Recruiting
Autologous very small embryonic-like stem cells (VSELs)	Estimated POF population not shown	-Clinical diagnosis of POF -Abnormal sex hormone levels	NCT03985462	Withdrawn
Human Adipose Derived Mesenchymal Stromal Cells	9 patients with POF	-Age: 20–39 -FSH >20	NCT02603744	Unknown
Human Adipose derived stem cells (ADSC)	4 patients with POF	-Age: 20–39 -Clinical diagnosis of POF -Lack of response to drug treatment -Willing to receive follow-up -Willing to conceive a baby	NCT01853501	Unknown
Human Umbilical Cord Mesenchymal Stem Cells (hUC-MSCs)	320 patients with POF	-Age: 20–40 -Clinical diagnosis of POF -Fertility requirement and sperms in couple	NCT03033277	Unknown
Human Umbilical Cord-derived Mesenchymal Stem Cells (hUC-MSCs)	23 patients with POF	-Age: 20–39 -Clinical diagnosis of POF -Lack of response to drug treatment	NCT02644447	Completed
Ovarian Stem Cells	11 patients with POF	-Age: 20–39 -Clinical diagnosis of POF, POI, or DOR -Early follicular phase FSH >15 IU/L -AMH <0.16 ng/ml or below the level of detection for the assay used -Undergoing ovarian biopsy by laparoscopy or clinically indicated abdominal surgery that provides access to the ovaries -Early follicular phase FSH >15 IU/L -AMH <0.16 ng/ml or below the level of detection for the assay used	NCT01702935	Completed

POF, premature ovarian failure; ESRHE, European Society of Human Reproduction and Embryology; FSH, follicle stimulating hormone; POI, premature ovarian insufficiency; DOR, diminished-ovarian reserve.

PROPOSED MECHANISMS FOR STEM CELL THERAPY

As it has been described, different studies showed that BMDSCs are effective for POI treatment in animals (and present promising results in humans) (103). To understand the underlying mechanisms would allow us to optimize these strategies and to find the optimal cohort of patients who will be benefited.

Overall, SCs show the ability to act in a paracrine manner thanks to the secretion of soluble factors and chemokines (75, 79, 81, 91). Paracrine action could help to restore damaged tissue, in this case the ovarian niche, by regulating different vital processes in this microenvironment. In this context, different studies show the involvement of BMDSCs in the regulation of angiogenesis, apoptosis, the regulation of the immune system, and fibrosis in the ovary (108) (**Figure 2**).

In the context of angiogenesis regulation in the ovary mediated by BMDSCs, the increase of vascularization in the ovarian niche, improve the healing process that occurs in a cyclic manner, and it may be beneficial for ovarian recovery (91). It has been reported that factors produced by these cells such as Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor-2 (FGF2) and Interleukine-6 (IL-6) promote arteriogenesis *in vitro* and *in vivo* (109). BMDSCs have been shown to promote angiogenesis *in vitro* through the α5 β1 receptor (110) and through Platelet Derived Growth Factor (PDGF) (111). In ovarian tissue, angiogenin produced by BMDSCs has been reported to play a positive role in angiogenesis after transplantation (112).

Regarding the antiapoptotic-promotion property of BMDSCs in GCs in the ovary (72, 81, 87, 95, 101), it has been reported that the coculture with BMDSCs decreases the levels of the proapoptotic proteins P21 and BAX and increase the levels of the proto-oncogene *c-myc* in GCs (100, 101). It has been observed that different cytokines present in the BMDSCs culture medium—VEGF, HGF, IGF-1—are able to decrease apoptosis of granulosa cells *in vitro* (113) and *in vivo* (87) and promote their proliferation (114); which suggests that the secretion of these growth factors may be underlying the apoptosis decrease found after BMDSCs in the ovary.

The immunomodulatory effects of BMDSCs have been tested *in vitro* and *in vivo* in different diseases (115, 116). The regulation of the balance between different populations of immune cells or between pro-inflammatory and anti-inflammatory cytokines mediated by BMDSCs could underlie this immunomodulatory effect (108). MSCs have been reported to have immunoregulatory properties in the ovarian niche by regulating populations of macrophages, regulatory T lymphocytes, and associated cytokines (117, 118). TNF-alpha has also been associated with the immunoregulatory function of human MSCs in the ovary (119). MSCs have been reported to reduce SOD dismutase in the ovary after transplantation, suggesting that the recovery of the ovarian niche could also be due to a regulation of oxidative stress in this microenvironment (108, 120).

In relation to fibrosis decrease, after BMDSCs transplantation, a decrease in collagen levels has been observed (121), suggesting that the mechanism of action of these cells in the recovery of ovarian damage could also involve an antifibrotic effect (79). In fact, BMSCs may inhibit fibroblasts proliferation and decrease the level of extracellular matrix deposition (108).This antifibrotic effect has been associated with certain soluble factors such as HGF, adrenomedullim and Basic Fibroblast Growth Factor (bFGF) (122).

As it is said, the paracrine action of BMDSCs in the ovarian niche has been demonstrated by injecting only soluble factors from the culture medium of the SCs, obtaining similar results as with SCs transplant (79). In fact, the ASCOT clinical trial in POR

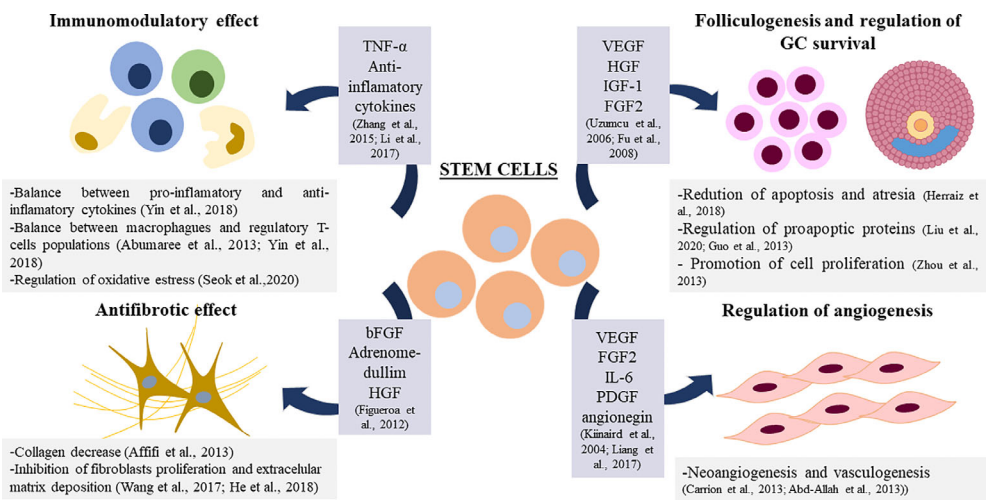

FIGURE 2 | Proposed mechanisms for Stem Cell Therapy in ovarian damage. TNF-α: Tumor Necrosis Factor α, VEGF: Vascular Endothelial Growth Factor, HGF: Hepatocyte Growth Factor, IGF-1: Insuline Like Growth Factor 1, bFGF: Basic Fibrolast Growth factor, FGF2: Fibrolast Growth Factor 2, IL-6: Interkuline 6, PDGF: Platelet Derived Growth Factor.

women highlighted the fact that aphaeresis provided relevant components for success, including specific BMDSC-secreted soluble factors, which acting in a paracrine manner promote growth of the already existing residual follicles in impaired ovaries. This study found that positive response was not limited to the injected ovary, as circulating SCs during the mobilization phase also reached the non-injected ovary producing an increase in the AFC in both sides (102). Furthermore, there are recent reports of nervous tissue rejuvenation and repair by injection of young growth factor enriched plasma, umbilical cord blood plasma, and plasma specific proteins into damaged and aged organisms (123–125).

This fact opens up new possibilities to combat damage in ovarian tissue such as the injection of soluble factors or platelet-rich plasma (PRP).

THE PRP APPROACH

PRP injection has been used for years in several fields of Medicine (orthopedics, sports Medicine, aesthetics, etc.) but in the context of assisted reproductive techniques (ART), intraovarian injection of autologous PRP has been recently proposed as an alternative to restore ovarian function in POI women (**Tables 4.1** and **4.2**). This approach is also based on the paracrine signaling, as PRP is a concentrate composed by platelet-enclosed growth factors, which could promote tissue healing, angiogenesis and cell growth (132, 133). Pantos et al. (129) introduced by first time this new approach without the direct use of SCs to reactivate folliculogenesis in perimenopausal women. In this study, the ovaries of eight perimenopausal women of advanced maternal age (41–49 years old) were infused with platelet-rich plasma by transvaginal ultrasound-guided injection. Treatment resulted in restoration of menses, with presence of ovarian follicles that allowed oocyte retrieval after IVF treatment in all patients and cryopreservation of 1.50 ± 0.71 embryos. However, a limitation of the study is that it only included eight women and did not document their previous ovarian reserves. These effects might be due to an increased in ovarian vascularization, with a key role in ovarian function as well as in promoting follicle development increasing follicular cell proliferation and survival (95). After this first report, ovarian and endocrine positive effects and live births have been also reported in several series of patients with impaired ovarian function such as POR (127) and POI women (130, 134).

Sills et al. (126) showed, in a study population of aged women (42 ± 4 years; infertility duration 60 ± 25) with diminished ovarian reserves, that intraovarian administration of PRP was able to induce an increase in serum AMH and a decrease in serum FSH, sufficient to permit oocyte retrieval (5.3 ± 1.3 MII) and blastocyst cryopreservation in all recruited patients 2 months after treatment.

The firsts controlled clinical trials involving a relevant number of patients with different and properly characterized ovarian phenotypes have been published in the last year. Sfakianoudis et al. (128) reported four pilot studies on POR, POI, perimenopausal, and menopausal women with a total of 120 participants recruited (n = 30 each). In the case of POR women (38.4 ± 2.0 y.o.), they found that PRP injection was able to improve ovarian reserve biomarkers, as AMH levels as well as AFC increased in the first and second menstrual and remained stable in the third while FSH and LH levels were reduced in the first menstrual cycle and remained stable. The main ICSI cycle outcomes were increased, especially the number of oocytes retrieved and the number of MII and embryos, all together with a reduction of the cancelation rate, a main concern in POR women. Overall, the reported clinical pregnancy rate for POR was 46.6% (14 out of 30 women) with 12 participants having a live birth.

TABLE 4.1 | Human studies involving PRP treatment for POR patients.

Study population	Infertility history	PRP preparation	Administration method	Main findings	Reference
4 patients with DOR (38–46 years old) AMH = 0.38 ng/ml ± 0.38 FSH = 13.6 mIU/ml AFC = 4 ± 0.8	Infertility duration 60 ± 25 months	Centrifugation and activation with calcium gluconate	Transvaginal ultrasound-guided ovarian stroma injection.	-AMH increase or/and FSH decrease in all cases -Oocytes retrieval (5.3 ± 1.3 MII oocytes) in all cases. -IVF occurred range 59–110 days after treatment. -At least one cryopreserved blastocyst for each patient.	Sills et al. (126)
23 PORs (34–40 years old) AMH <0.5–1.1 ng/ml	Infertility duration 0.5 ± 3.77 years	Blood Transfusion Organization standard method	Transvaginal ultrasound-guided ovarian injection	-Oocyte retrieval increase (2.1 *vs* 0.64 before treatment). -2 spontaneous conceptions. -3 live births.	Farimani et al. (127)
120 patients. -30 POR patients (38.40 ± 2.01) AMH = 0.66 ± 0.20 ng/ml; FSH = 10.71 ± 1.62 IU/ml; AFC = 2.63 ± 0.93*	Infertility duration: -5.82 ± 1.02 years (POR group)	RegenACR- C Kit	Transvaginal ultrasound-guided intramedullar ovarian injection	-POR patients: AMH and AFC increased and FSH decrease two menstrual cycles after treatments. 14 pregnancies and 12 live births.	Sfakianoudis et al. (128)

*POR, poor ovarian responder; DOR, diminished ovarian reserve; AFC, antral follicle count; AMH, anti-mullerian hormone; FSH, follicle-stimulation hormone; COS, controlled ovarian stimulation; *reported only for those women with positive response after PRP.*

TABLE 4.2 | Human studies involving PRP treatment for POI and perimenopausal and menopausal patients.

Study population	Infertility history	PRP preparation	Administration method	Main findings	Reference
8 perimenopausal women with idiopathic POI (41–49 years old). Baseline characteristics not reported.	Amenorrhea duration 4.88 ± 1.13 months	RegenACR- C Kit	Transvaginal ultrasound-guided ovarian injection.	-Menstrual cycle restoration 1–3 months after treatment. -Follicle development and oocyte retrievals in all cases, (1.50 ± 0.71 MII oocytes.) -1.50 ± 0.71 resultant embryos -Cryopreserved transfer has been performed.	Pantos et al. (129)
2 women with POF (47 and 27 years old) and a menopausal woman (46 years old). AMH 0.06–0.17 ng/ml; FSH 46.5–119 mIU/ml; AFC = 0	Amenorrhea duration 12 months (menopausal patient), not reported (POF patients)	RegenACR- C Kit	Transvaginal ultrasound-guided ovarian injection	-Menstrual cycle restoration in all cases 1–2 months after treatment. -AMH increase or/and FSH decrease in all cases -Pregnancy in natural conception through natural conception 2–6 months after treatment.	Pantos et al. (130)
1 woman with premature menopause (40 years old) AMH = 0.02 ng/ml; FSH = 149 mIU/ml	Amenorrhea duration 19 months	RegenACR- C Kit	Transvaginal ultrasound-guided intramedullar ovarian injection	-Menstrual cycle restoration 6 weeks after treatment. -FSH decrease and slightly AMH increase. -Biochemical pregnancy, resulting in a spontaneous abortion at the 5th week of pregnancy.	Sfakianoudis et al. (126)
120 patients. -30 patients with POI (35.9 ± 1.9 years old); AMH = 0.18 ± 0.04 ng/ml; FSH = 40.611 ± 6.05 IU/ml; AFC = 0* -30 perimenopausal women (43.4 ± 1.4 years old). AMH = 0.96 ± 0.28 ng/ml; FSH = 18.51 ± 2.62 IU/ml; AFC = 1.54 ± 0.51* -30 Menopausal women (48.8 ± 1.6 years old). AMH= 0.13± 0.03 ng/ml; FSH= 80.27 ± 5.03 IU/ml; AFC=0 *	Amenorrhea duration: -16 ± 2.42 months (POI group) -15.69 ± 1.75 months (Menopausal group)	RegenACR- C Kit	Transvaginal ultrasound-guided intramedullar ovarian injection	-POI patients: menses recovery and FSH increase in 60% of patients. 3 pregnancies and 3 live births. -Perimenopausal patients: menses recovery and FSH increase in 80% of patients. 4 natural pregnancies and 3 live births. -Menopausal patients: menses recovery and FSH increase in 43.3% of patients. 1 pregnancy and 1 live birth.	Sfakianoudis et al. (128)
311 patients with POI (34.6 ± 4.0 years old) AMH = 0.01–0.82 ng/ml FSH = 25–155 mIU/ml** AFC = 1.26 ± 0.8	Infertility duration 6.8 ± 4.9 years	Centrifugation and T-lab autologous platelet-rich plasma kit (T-Biotechnology Laboratory)	Transvaginal ultrasound-guided intramedullar underneath ovarian cortex injection	-Spontaneous pregnancy in 7.4% of patients (69.6% achieved live birth). -AMH and AFC increased after treatment. FSH increase not observed. -Antral follicle observation and COS initiation in 64.6% of patients. 40.8% of these patients achieved at least one blastocyst. -22.8% of stimulated patients achieved a pregnancy after transfer.	Cakiroglu et al. (131)

POI, premature ovarian insufficiency; POF, premature ovarian failure; AFC, antral follicle count; AMH, anti-Mullerian hormone; FSH, follicle-stimulation hormone; COS, controlled ovarian stimulation.

**Reported for women with positive response after PRP; ** Reported only for those women achieving pregnancy after PRP.*

For the other three pilot studies included, the reported primary outcomes were different according to the diagnosis of the recruited patients, as for menopause and POI women menses recovery and FSH levels became a principal result. In the POI population (35.9 ± 1.9 y.o.), they observed that 18 women (60%) positively responded to PRP treatment when considered as menstrual cycle restoration and reduced FSH levels, with a total of three pregnancies and three live births (PR:10%). These results slightly improved in the perimenopausal women (43.4 ± 1.4 y.o.), where 24 women (80%) positively responded to PRP treatment. For these women menstrual cycle regulation as well as FSH level reduction was observed having four natural conceptions and three live births (PR:13.3%). Finally, in the menopausal group (48.8 ± 1.6 y.o.), 13 women (43.3%) positively responded to PRP treatment with one pregnancy and one live birth (1%).

To date, the largest study has been developed by Cakiroglu et al. (131), in a population of 311 women (34.8 ± 4.3 y.o.) with POI diagnosis based on the ESHRE criteria. After intraovarian injection of autologous PRP, the 7.4% of POI women (23/311) achieved a spontaneous pregnancy one or two menstrual cycles after treatment

with 7 miscarriages and 16 live births reported. From the remaining patients, development of at least one antral follicle was noticed in 201 allowing the initiation of controlled ovarian stimulation from the second to the sixth menstrual cycles after intervention, although oocyte retrieval was only achieved in 130 and MII-oocytes obtained in 93 women. The 40.8% of stimulated women obtained at least one cleavage stage embryo scored as A/B according to morphological criteria. To date, only 57 of these patients underwent embryo transfer (both fresh or frozen) as the remaining ones having embryos decided to cryopreserve them for a later transfer. A total of 13 achieved a pregnancy after ET (22.8%) although 4 experienced a miscarriage. Nevertheless, FSH levels did not improve after treatment when compared to previous values, although AMH and AFC increased. Overall, this study reported a total of 36 pregnancies in 311 women (11.5%PR) and 8% of live birth rate or sustained implantation, which although opening a new path for the management of POI women. It is relevant for the overall evaluation of these rates to highlight that at the moment of publication several patients still had their embryos cryopreserved for future transfer.

All together, the studies evaluating PRP ovarian injection are encouraging as they open a new path to a clinical alternative more easily applied than the stem cell based therapies as ovarian injection is performed in a similar intervention to oocytes collection. Nevertheless, their results should be evaluated with caution as for now there are no experimental studies evaluating the wide spectrum of PRP effects, duration, and mechanism in the ovarian tissue, and the reported human studies lack from an adequate control group to properly establish the efficiency of the technique. Thus, results of placebo in double blinded randomized clinical trials should be obtained and carefully evaluated before proposing PRP as a routine treatment for POI and DOR patients in ART clinics. Furthermore, it is important to bear in mind that POI pregnancy rates across studies ranged from 2.2 to 14.2% and spontaneous resumption of ovarian function occurs in 25% of patients, and primordial and pre-antral follicles are frequently found in ovarian biopsies from women diagnosed as having POI (8).

CONCLUSIONS

This field of investigation opens new opportunities for ovarian rescue in women with impaired ovarian reserve, such as POR and POI patients, by different strategies focused on the rescue of already existing follicles. These approaches include the inhibition of molecular pathways by IVA and tissue mechanical fragmentation, stem cell administration, and PRP ovarian injection. Although heterogeneous, all the techniques have a common characteristic, to promote growth of follicular cells by activating different paracrine signaling mechanisms. This finding is of paramount relevance for the future design of feasible and less invasive clinical options. Nevertheless, these proposals should be previously supported by comprehensive experimental and mechanistic studies. The inclusion of a proper control group should be mandatory in future randomized clinical trials for a realistic evaluation of the technique's efficacy in selected group of patients.

AUTHOR CONTRIBUTIONS

AP has performed a bibliography search and analysis and she has written the manuscript. JG-V has critically revised the manuscript, and he has coordinated the study. SH has performed a literature search and analysis, she has written and critically revised the manuscript, and she has coordinated the study. All authors contributed to the article and approved the submitted version.

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REVIEW
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In Vitro Follicular Activation and Stem Cell Therapy as a Novel Treatment Strategies in Diminished Ovarian Reserve and Primary Ovarian Insufficiency

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Usually poor ovarian response (POR) to gonadotropins reflects a diminished ovarian reserve (DOR) that gives place to few recruitable follicles despite aggressive stimulation. The reduction in the quantity and quality of the oocytes with advanced age is physiological. However, some women experience DOR much earlier and become prematurely infertile, producing an accelerated follicular depletion towards primary ovarian insufficiency (POI). Up to now, egg donation has been commonly used to treat their infertility. In the last thirty years, specialists in assisted reproduction have focused their attention on the final stages of folliculogenesis, those that depend on the action of gonadotrophins. Nevertheless, recently novel aspects have been known to act in the initial phases, with activating and inhibiting elements. *In vitro* activation (IVA) combining the *in vitro* stimulation of the ovarian Akt signaling pathway in ovarian cortex fragments with a method named Hippo-signaling disruption. Later, a simplification of the technique designated Drug-Free IVA have shown encouraging results in patients with POI. Another innovative therapeutic option in these patients is the infusion of bone marrow-derived stem cells (BMDSC) in order to supply an adequate ovarian niche to maintain and/or promote follicular rescue in patients with impaired or aged ovarian reserves. In this review, for the first time, both therapeutic options are addressed together in a common clinical setting. The aim of this review is to analyze the physiological aspects on which these innovative techniques are based; the preliminary results obtained up to now; and the possible therapeutic role that they may have in the future with DOR and POI patients.

Keywords: in vitro activation, primary ovarian insufficiency, poor ovarian reserve, Hippo signaling, stem cells, poor ovarian response, extracellular matrix, mechanobiology

INTRODUCTION

The ovarian reserve reflects the total of ovarian follicles including non-growing follicles (NGFs) together with those that are growing recruited in the preantral and antral stages phases that can finally reach ovulation. Women are born with a finite pool of ovarian follicles that decreases dramatically during intrauterine life from a peak of about 7 million to 1 million at birth. During childhood the descent continues, so that at the age of menarche about 400,000 persist follicles. Finally at menopause there are only less than 1,000 follicles in the ovaries (1, 2). Moreover, as follicle numbers gradually decline with age, thus a sequence of reproductive events occurs, beginning with reduced fecundity and natural sterility, progressing through menstrual cycle irregularity towards a complete cessation of menstruation at menopause. In theory, this sequence unfolds according to "fixed time intervals" before the subsequent stage (3, 4).

According to these concepts, three different scenarios may occur: a normal decrease of ovarian reserve with age, a lower ovarian reserve set prenatally with an usual postnatal decay, or a decrease of ovarian reserve during adverse postnatal environmental or nutritional challenges (5). Anyhow, the diminished ovarian reserve (DOR) constitutes one of the most important therapeutic challenges in assisted reproduction, since the ovarian response to gonadotropin stimulation is an essential prognostic factor (2, 6, 7).

Primary ovarian insufficiency (POI) that affects 1% of women would be the most extreme clinical manifestation of accelerated ovarian follicular depletion and whose only available reproductive treatment is egg donation (8). Even though menstrual cycles cease in these patients, some of them still contain small residual ovarian follicles.

Recently, due to new physiological knowledge in the earliest folliculogenesis phases, attention has been focused on the possibility of activating dormant follicles in patients with POI.

On the one hand, it has been shown that the balance between activating (Akt stimulatory) and inhibitory pathways (Hippo-signaling) is crucial (9). Moreover it has been demonstrated that the manipulation of these mentioned pathways can have clinical application, such as the disruption of the Hippo-signaling pathway by fragmenting ovarian tissue and activating it by incubating with Akt stimulants in primary ovarian insufficiency patients (POI) (10–12). That technique is named ovarian *in vitro* activation (IVA). Overtime, a modification of the technique has been reported aiming to the disruption of the Hippo-signaling pathway alone and renouncing to the chemical activation of the ovarian tissue (Drug-Free IVA) (13–15).

On the other hand, it has been suggested that infusion of human-derived stem cells could supply a fitting ovarian niche to maintain or promote follicular rescue in patients with impaired or aged ovarian reserves. Human studies propose bone marrow-derived stem cells (BMDSC) both mesenchymal and hematopoietic are feasible candidates to promote ovarian rejuvenation (16, 17).

Follicular waves, quality oocytes and live births were obtained from residual follicles with the IVA approach and BMDSC infusion. Still, more studies are necessary to define the real role of these therapeutic options in patients with DOR and POI.

INTRAOVARIAN CONTROL OF EARLY FOLLICULOGENESIS

Ovarian follicular reserve is conditioned by the periodic sequence of the "activation" of primordial follicles that leave their quiescent state. Primordial follicle activation involves recruitment of primordial follicles into folliculogenesis for the eventual selection of one oocyte for ovulation. When this activation is accelerated abnormally the ovarian reserve can be seriously affected (18).

Current strategies for controlled ovarian stimulation focus their effect on growing follicles, whereas dormant primordial follicles cannot be activated by known ovarian stimulation protocols. The knowledge of the biological bases for the awakening of the primordial follicles is essential however, up to this point they are little known. Multiple local factors and intracellular signaling pathways are involved. Activators such as BMP4/7, GDF-9, KIT-ligand, FGF2/7, insulin, GREM1/2, LIF and suppressors factors (AMH, LHX8, PTEN, Tsc1m/TORC1, FOXO3a, YAP/Hippo-signaling, and FOXL2) have been reported to be related to primordial follicle development (9, 19)

Most of the signaling networks and molecules involved in primordial follicle activation have been studied using rodent "lost-on-function" models (20, 21). Currently it is considered that in cases of POI the depletion of ovarian reserve is produced by an exaggerated acceleration of the activation of the pool of primordial follicles.

The maintenance of a correct ovarian reserve will depend on a balance between activating and inhibiting factors. In this sense, recent studies have focused their attention on the phosphatase and tensin homolog (PTEN)/phosphatidylinositol-3-kinase (PI3K)/protein kinase B (AKT)/forkhead box O3 (FOXO3) and Hippo-signaling pathway.

Although the complete mechanism of follicular activation remains undeciphered, studies conducted in mice knockout, have shown that the specific deletion in oocytes of the PTEN and Foxo3 gene promote the activation and the growth of all primordial follicles (22, 23). The PTEN gen encodes a phosphatase enzyme that negatively regulates the PI3K-Akt-Foxo3 signaling cascade (24, 25). It has also managed to promote the activation of primordial follicles sleepers using PTEN inhibitors and/or activators Akt, both in murine and human ovaries. In the ovaries, AKT is a prominent kinase in the PI3K/AKT/mTOR pathway and is expressed in both oocytes and granulosa cells of human follicles (25, 26). AKT has a wide range of substrates with both direct and indirect roles in follicle activation (24, 25).

Coordination of cell proliferation and death is essential for the maintenance of organ size and tissue homeostasis during postnatal life. In mammals, the coordination of both processes is orchestrated by Salvador/Warts/Hippo signal. This signaling pathway consists of different regulators negative effects that act in a cascade of kinases that in ultimately antagonizes the

transcriptional coactivator Yes Associated Protein (YAP) and its PDZ-binding motif (TAZ) inducing a suppression of growth (26).

YAP is inactivated by phosphorylation mediated by the Hippo pathway, which excludes it from the nucleus, while the loss of Hippo signaling promotes the accumulation of YAP in the nucleus and an increase in its activity. Once inside the nucleus, the YAP protein acts in coordination with the TAZ transcriptional activators to trigger the expression of CCN growth factors and inhibitors of apoptosis BIRC (baculoviral inhibitors of apoptosis repeat containing). This results in increased proliferation and cell growth. Unlike most activated signaling pathways by extracellular ligands, Hippo is regulated by a network of components related to adhesion, shape and cell polarity (27). These cellular features are mediated by rapid changes in polymerization of the globular actin (form G) to filamentous actin (form F) which induced by fragmenting the tissue and which are the Hippo pathway inhibition triggers. This way is implicated for organ size control, and recent studies provide the theoretical basis to disrupt this pathway that can promote follicle growth.

Studies have shown the deletion of the SAV1 or MST gen in hepatocytes results in augmented livers in mice (28, 29). In cardiomyocytes, deletion of SAV1 leads to enlarged hearts (30) and Hippo-signaling is also engaged in tissue regeneration and expansion of stem cells (31).

Based on initial observations, it has been demonstrated that mechanical signals as ovarian fragmentation, and other forms of ovarian injury led to actin polymerization that disrupt ovarian Hippo-signaling, resulting in nuclear translocation of YAP. Nuclear YAP interacts with transcriptional enhanced associate domain (TEAD) producing transcriptional factors to increase the expression of downstream biochemical signals (cysteine-rich 61, connective tissue growth factor and nephroblastoma overexpressed (CNN) growth factors and baculoviral inhibitors of apoptosis repeat containing (BIRC) apoptosis inhibitors. All this leads to follicle growth (9, 32).

In summary, selective primordial follicles develop to the primary stage under the control of AKT and mTOR signaling (initial recruitment), whereas most primordial follicles remain arrested by dormancy factors. Development of preantral and antral follicle is restrained by the inhibitory Hippo-signaling pathway. Therefore, correct folliculogenesis and maintenance of the ovarian follicular reserve depend on an adequate balance between these two routes.

MECHANICAL SIGNALING IN OVARIAN FUNCTION

Although many hormonal and molecular factors have provided great information about the initial phases of folliculogenesis, the role of the extracellular matrix (ECM) in these processes remains undefined. The role of mechanobiology in the knowledge of ovarian function and the dynamic reciprocity that exists between ovarian cells and their microenvironment has recently gained great interest (33, 34). It is known that while the localization of primordial follicles in the collagen-rich ovarian cortex offers a rigid physical environment that supports follicle architecture and probably plays a role in their survival, ovarian ECM rigidity limits follicular development and therefore oocyte maturation, keeping the primordial follicles in their quiescent stage (35). Besides, it has been shown that growing follicles migrate to the medulla of the ovary, where they encounter a softer, more pliant ECM, allowing its maturation. Thus, changes in the rigidity of the ovarian ECM have a direct effect on follicle development.

The relationship between ovarian physical environment and its functionalism has been demonstrated in some pathological situations, such as primary ovarian insufficiency (POI) and polycystic ovary syndrome (PCOS).

Studies have shown that the distribution of primordial follicles varies depending on the age of the patients. In early ages they would be fundamentally located in the most superficial part of the ovarian cortex. In patients with POI, the few existing primordial follicles would be located very close to the medulla (36). Moreover, in patients with PCOS it has been demonstrated that all pre antral follicles are trapped in cortex which could be explained by an aberrant Hippo-signaling, considered important for adequate follicular growth (37).

Finally, and taking into account all of the above, a recent study in which the remodeling of the mechanical components of the matrisome was investigated has shown that the primordial follicles are located in areas of the ovarian cortex rich in collagen, conferring a rigid physical environment that supports follicle architecture and limits follicle expansion. In addition, the matrisome components vary depending on age and on the different stages of follicular maturation (38).

CONVENTIONAL *IN VITRO* ACTIVATION (IVA) AND DRUG-FREE IVA

Mechanobiology is an emerging field of science based on studying how physical forces and changes in the mechanical properties of cells and tissues are able to regulate their proliferation and differentiation. This scientific discipline attempts to explain how mechanical forces critically regulate cellular biochemistry and gene expression as well as tissue development (39). This effect is established by mechanisms of mechanotransduction. Mechanotransduction, the process by which cells sense and respond to mechanical signals, is mediated by extracellular matrix and cytoskeletal structures. Hippo-signaling pathway is basic in mechanotransduction (40, 41).

Experimental studies in mouse ovary demonstrated that using a PTEN inhibitor and a PI3K activator for two days an increase of FOX3 is produced in oocytes of primordial follicles, suggesting a follicular activation. Subsequent transplantation of these ovaries into ovariectomized hosts revealed the presence of preovulatory follicles and mature oocytes (42). Later studies showed that the fragmentation of the ovarian tissue produced a polymerization of actin and a disruption of the Hippo pathway,

favoring follicular growth and the retention of mature oocytes (12, 42)

Considering these experimental studies, conventional IVA was established combining Akt stimulators treatments, ovarian fragmentation and autografting cortical strips by laparoscopic surgery in POI patients (10). After ovarian stimulation, retrieval of mature oocytes, IVF and embryo transfer, a healthy baby was delivered. Conventional IVA was successfully used in POI patients in Japan (11) and China (43).

Despite the encouraging results obtained with this technique, it has been hampered by some aspects, mainly the need to perform two laparoscopies and by the possible harmful effect of stimulant substances (44). In this line, a recent modification of the technique has been reported aiming to focus only on the disruption of the Hippo-signaling pathway and avoiding the chemical activation of the ovarian tissue (Drug-Free IVA) (**Figure 1**). Fabregues et al. reported a pregnancy for the first time using this technique (13). Another case reported a new pregnancy with this technique (45) and in a large series only a biopsy/scratch of the ovarian cortex was performed, observing follicular waves in 20% of the patients (46). The largest series of patients with POI to whom this technique was applied reported follicle development in 50% of them with oocyte retrieval in five of the 14 patients with four successful pregnancies with a pregnancy rate of 57% per oocyte retrieval and 67% per embryo transfer (14).

Furthermore, Drug-Free IVA has been used preliminarily in POR patients based on the hypothesis that these still have multiple residual follicles that could be activated by mechanical stress. One recent study observed in nine of 11 patients with POR treated with drug-free IVA, multiple growth waves and increases in antral follicle counts were detected after ovarian stimulation treatment. Later retrieval of mature oocytes for IVF allowed 16 embryo transfers in five patients, leading to one live birth, two ongoing pregnancies and one miscarriage. Another patient conceived naturally (15).

In contrast, another recent study has questioned the efficacy of Drug-Free IVA in patients with POR. In 20 patients who were followed up for 10 weeks after the procedure, no significant increase in AFC and AMH levels was observed. Despite this fact, 12 of these patients achieved subsequent pregnancies, which probably reveals a limitation of the study in terms of correct follow-up to assess long-term follicular activity after ovarian cortical fragmentation, followed by autologous grafting under laparoscopic surgery (47). The results achieved so far with IVA and Drug-Free IVA are summarized in **Table 1**.

OVARIAN NICHE CONCEPT

Researchers have studied the structure and functions of the stem cell niche in the hematopoietic system, intestinal system, neuronal cells, spermatogonia and the ovary system (48). The stem cell niche is the micro-environment where the surrounding stem cell survive, and it is composed of niche cells, matrisoma, and cytokines. For many years it has been considered that the aging of the organs is due to the senescence of the stem cells (49, 50). However, some authors indicate that the aging problem is more connected with the aging of the stem cell nests (51, 52). Specifically, in the ovary, ovarian stem cell nest is mainly composed of granulosa cells, vascular endothelial cells, immunologically relevant cells and molecules, surrounding the ovarian reproductive stem cells and regulating their functions. Ovarian germ stem cells belong to the category of adult stem cells and most authors suggest that there are two mechanisms that explain stem cell aging. On the one hand, endogenous aspects that would directly affect them and, on the other, other elements that would be more important that would focus on the exogenous microenvironment. All this is still to be elucidated.

In other words, we know that in increasing age, ovarian function cannot be maintained, despite the ovarian germ stem cell activity and several experimental studies indicated that

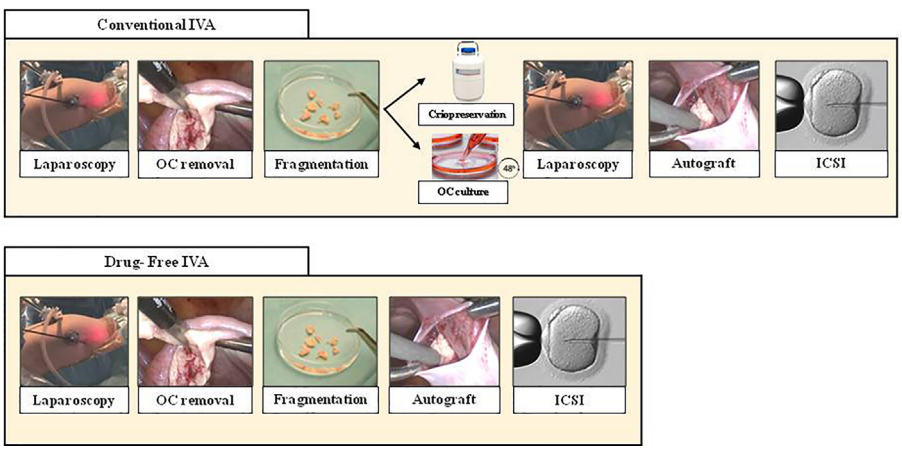

FIGURE 1 | Comparison between Conventional IVA (top line) and Drug Free IVA (bottom line). As seen above, Drug-free IVA avoid second laparoscopy and culture ovarian tissue. OC, ovarian cortex; ICSI, intracytoplasmic sperm injection.

TABLE 1 | Human studies involving IVA and Drug-Free IVA in POI and POR patients.

Author	Ref.	Procedure type	No. of patients	Inclusion criteria	Follicle development/Total	Pregnancies/ Total	Live birth: total
Kawamura et al., 2013; Suzuki et al., 2015	(10, 11)	IVA	37	POI	9/37	3/37	2:37
Zhai et al., 2016	(43)	IVA	14	POI	6/14	1/14	1:14
Pellicer et al., 2017**	–	OFFA (Drug-Free IVA)	14	POI	–	3/14	3:14
Zhang et al., 2018	(46)	Biopsy/Scratch	80	POI	11/80	1/80	1:80
Fàbregues et al., 2018; Ferreri et al., 2020	(13, 14)	Drug-Free IVA	14	POI	7/14	4/14	4:14
Mahajan et al., 2019	(45)	Drug-Free IVA	1	POI	1/1	–	–
Kawamura et al., 2020	(15)	Drug-Free IVA	11	POR	9/11*	5/11	2:11 2 ongoing 1 miscarriage

Patients increased AFC.

***Unofficial data are from conference presentations of stated scientist.*

POI, Primary ovarian insufficiency; IVA, in vitro activation; OFFA, Ovarian fragmentation for folicular activation.

ovarian function decline is mainly related to the aging of the ovarian germ cell nests but not to the aging of the ovarian germ stem cells (53, 54). All these concepts would serve as a basis to propose the improvement of the niche as a method for the rejuvenation of the ovary.

ADULT STEM CELL BASED THERAPIES TO PROMOTE FOLLICLE DEVELOPMENT

In recent years, there has been a particular interest in adult stem cell therapy, with the potential to provide the right environment for oocyte development from quiescent primordial follicle. In the human body, the different organs and tissues will present a different proportion of adult stem cells, depending on their turnover. In tissues with high cell turnover, such as the marrow bone or intestine, the population of stem cells is high, and they are usually active throughout life. In this way they are available for repair of these fabrics in great demand. There are other tissues with cell turnover inferior, and where these stem cells remain quiescent and are only activated in the event of injury.

Regarding adult stem cells, research has focused on adult stem cells of hematopoietic origin and of mesenchymal origin. Mesenchymal stem cells (MSCs), relative to tissue cellular, have a very high replication capacity. On the other hand, they have the potential to differentiate themselves when they are cultivated *in vitro* to other cells, such as osteocytes, adipocytes, and chondrocytes (55, 56).

Bone marrow-derived stem cells (BMDSC) are mononuclear cells with low immunogenicity, which makes them ideal for therapy and transplantation. They can renew themselves, to maintain the population of stable stem cells, but they also may have the ability to differentiate from other tissues (adipocytes, cartilage, etc.). These cells can migrate to other damaged tissues and differentiate, after being induced by the release of cytokines from damaged tissues. Yet, they liberate themselves cytokines and growth factors, which promote anti-apoptosis and antifibrosis to restore the ovary, such as VEGF (vascular endothelial growth factor), IGF-1, and HGF (hepatocyte growth factor) (57). Animal studies demonstrated that BMSCs play an important role in restoring injured ovaries in POF induced by chemotherapy in rats (58). Moreover, they also restore ovarian hormone production and reactivate folliculogenesis in a mouse model of POF caused by cisplatin (59, 60). Many researchers have studied whether is possible to regenerate ovarian function. As it has been previously mentioned, adult stem cells have a regenerative capacity through paracrine release of soluble factors: e.g. cytokines, chemokines, growth factors. Thanks to this mechanism, they could take part in the regeneration of dysfunctional ovaries (61, 62). In fact, the soluble factors described in stem cells had already been related to folliculogenesis in ovaries with normal function. Among the growth factors released physiologically by stem cells we find IGF-1, TGF-ß (Transforming growth factor ß), EGF (Epidermal Growth Factor), and FGF1 and FGF2 (Fibroblast Growth Factor1 and 2). FGF-2 has been associated with a positive response to a stem cell therapy, thanks to its action on granulosa cells (63). Given their pluripotency and low immunogenicity, BMSCs are believed to have therapeutic potential for POI.

PRACTICAL APPLICATION OF BMDSC THERAPIES

Clinical cases suggested the possible role of stem cells in the recovery of ovarian function. Recovery of fertility and endocrine function of the ovary, after bone marrow transplantation (BMT), in patients with iatrogenic POI after chemotherapy for malignancies has been reported. These cases describe, in addition, spontaneous pregnancies in patients who presented iatrogenic menopause from myeloablative chemotherapy administered in this type of disease.

Hershlag et al. published four cases of induced early menopause after chemotherapy or radiotherapy. All patients

received as adjunctive treatment for their disease in addition autologous stem cell transplantation. Two of them with Hodgkin's Lymphoma and two with infiltrating ductal carcinoma breast, which prior to treatment for their disease conserved regular menses. After treatment, all of them started with amenorrhea and climacteric symptoms, compatible with POI. The authors published spontaneous pregnancies in the 4 patients after having been diagnosed with POI, probably a consequence of autologous transplantation of stem cells (64). Five years later, Veitia et al. reported another case, where a woman regained fertility and achieved spontaneous pregnancy after having been diagnosed with POI of iatrogenic cause. This patient, with Fanconi anemia, received myeloablative chemotherapy and radiotherapy following by an HLA-compatible allogeneic hematopoietic transplant. Years later, it was evidenced a return of menstruation and a spontaneous pregnancy with subsequent birth of a premature (currently healthy) baby. After analyzing the DNA of the baby, that of the mother and that of the donor, it was confirmed the genetic compatibility between the mother and the baby, ruling out the existence of a genetic relationship between the donor and the baby. This makes it quite unlikely that stem cells will produce a replacement direct from the oocytes, if not through the regeneration of the ovarian niche (65).

The first study in which the effect of BMCSD in human ovarian tissue was analyzed as a potential treatment in patients with POI and POR was performed by Herraiz et al. BMCSD were obtained by apheresis after treatment with G-CSF (granulocytic colony stimulating factor) from patients with this diagnosis. These cells were infused in two different groups of immunosuppressed mice, which had been reduced ovarian reserve with different doses of chemotherapy, using more dose to mimic patients with POI, and lower dose to mimic patients with POR. These mice had previously undergone a xenograft human ovarian tissue. In this population of mice, a long-term recovery of fertility was reported, thanks to an increase in ovarian vascularization, proliferation of cells at the level of the ovarian and follicular stroma and a reduction of follicular apoptosis (66).

The preliminary results obtained with the use of stem cells in POI patients seem to depend on two aspects: the stem cells source and the method of administration to reach the ovary. Although adipose-derived stem cells and umbilical cord stem cells have been used in experimental studies, those derived from bone marrow (BMSCs) have aroused greater interest. The main reasons are its low immunogenicity, the ease of being isolated and amplified *in vitro*, and its paracrine and immunomodulatory functions. They migrate to the site of injured tissue and differentiate into specific cell types in the tissue under the induction of certain factors to reconstruct the local micro-environment. By enhancing the function of endogenous cells and regulating the immune response, they are involved in the repair of tissue damage, which makes BMSCs an ideal seed cell for transplantation.

Regarding the administration techniques, human stem cells have been infused into one or both ovaries by various methods, such as transvaginal ultrasound-guided injection (16), ovary injection *via* laparoscopy intra-arterial catheterization of the ovarian artery (67) or a combination of techniques (68, 69). Further studies are needed to determine the most effective approach, although less invasive methods are required for both stem cell collection and instillation.

Gupta et al. described the birth of a live newborn in a 45-year-old postmenopausal woman after applying this technique. They injected the cells derived from bone marrow in the ovary by laparoscopy, infusing them into both ovaries. This way, the patient was exposed to two invasive techniques, in a first place to the puncture of the iliac crest and in a second instead of laparoscopy. After showing follicular growth in both ovaries, they started an ovarian stimulation and with this the pregnancy and subsequent live birth were achieved. They were the first researchers to report a live birth after stem cell therapy in a 45-year-old patient (67). In the same way, Edessy et al. also injected autologous BMSCs opting for the laparoscopic approach. This technique was carried out in 10 patients with POI, with an age between 26 and 33 years, with continued comprehensive checks for one year. The results, likewise, were promising, with the return of menstruation in two patients, one pregnancy, and a healthy live newborn (16).

Gabr et al. used autologous bone marrow mesenchymal cells in 30 women with idiopathic POI, with a range of very broad age between 18 and 40 years. Again, to obtain cells, a medullary aspiration was performed from the iliac crest. In order to introduce them into the ovary, they applied two different techniques in two groups of patients. In the first group, the cells were injected laparoscopically, while in a second group they were injected by catheterization of the ovarian artery (to access one of the two ovaries). After this technique, it was obtained a hormonal improvement in up to 86.7% of the patients, with a durable effect up to two years. Even though 3 patients underwent cycles of IVF, a gestation was achieved, obtained naturally (8).

In a recent and exhaustive study Herraiz et al. evaluated the effect of autologous stem cell ovarian transplant (ASCOT) on ovarian reserve and IVF outcomes of POR patients. The study consisted of BMDSC mobilization into peripheral blood by granulocyte colony stimulating factor (G-CSF) treatment and subsequent collection by apheresis. Cells were injected into the ovarian artery by intra-arterial catheter in one side in such a way that the contralateral ovary was considered as a control. ASCOT resulted in a significant improvement in AFC two weeks after treatment. When optimal ASCOT was considered (increase in AFC of three or more follicles and/or two consecutive increases of AMH levels), ovarian activity improved in 81.3% of women. ASCOT increased the number of stimulable antral follicles and oocytes. However, the embryo euploidy rate was low (16%). Five pregnancies were achieved. Two after embryo transfer and three spontaneously. Interestingly, this study evaluated soluble growth factors after apheresis secreted by circulating BMDSC, and positive effects were associated with the presence of fibroblast growth factor-2 (FGF)-2 and, thrombosponding (THSP)-1. These findings suggest that the treatment with only G-CSF could be effective and to be valid as an alternative therapy,

since it is a treatment much less invasive than ASCOT, and therefore with fewer side effects and greater safety for the patient. In the future, the use of these stem cells should be focused on the possibility to release soluble factors and their effect on the ovarian niche (68).

CONCLUSION

The study of the factors determining ovarian aging and the pathophysiological mechanisms involved constitutes one of the most interesting challenges of reproductive endocrinology. Knowledge of activating and inhibitory pathways in the early phases of folliculogenesis, as well as the concepts related to stem cell niche in the ovary, represent a new therapeutic scenario in patients with DOR and POI.

Data from animal experimentation has allowed the application of techniques such as IVA and Drug-Free IVA in patients with POI and DOR, reporting several live births. However, in order to confirm these encouraging results randomized studies are needed and therefore should still be considered experimental. The future challenges of these techniques should be focused on predicting which patients would be subject to treatment, since according to the current results only 50% of patients with POI would be able to achieve follicular waves. To know biomechanical aspects of the ovarian tissue of these patients and also to improve aspects of the surgical technique that can make mechanotransduction mechanisms more effective.

Future studies on the physical environment surrounding follicles in diseased ovaries are important to design more refined treatments. In this line, with future adaptation of intravaginal near-infrared cameras and improvement of IVA procedures, it would be possible to select patients with POI and patients with DOR with residual preantral follicles. For a better diagnosis of patients, recent advances in *in vivo* imaging tools could allow monitoring primary to secondary follicles

BMDSCs could be an alternative in ovary regeneration and follicular development in POR or POI patients. ASCOT approach involving the whole BMDSC population seems to be a good approach to treat POR women. This procedure has opened the possibility of proposing stem cell therapy simplified by BMDSCs mobilization into blood by granulocyte colony-stimulating factor (G-CSF) treatment avoiding laparoscopy or intra-arterial catheterization.

These new therapeutic options might soon become a reality.

AUTHOR CONTRIBUTIONS

FF conceived, structured, and wrote the text. All authors contributed to the article and approved the submitted version.

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Can Inhibin B Reflect Ovarian Reserve of Healthy Reproductive Age Women Effectively?

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Objective: The reference range and potential value of inhibin B are still unclear and controversial. This study aimed to define the variation trend of inhibin B in healthy women with age and explore its value in the reflection of ovarian reserve.

Methods: A total of 2524 healthy reproductive age women from eight medical institutes nationwide were recruited. The variation tendency of inhibin B with age was primarily established in the first group of 948 women and validated in another 605. We evaluated the relationship between inhibin B and classic ovarian reserve and function markers. The potency of inhibin B in predicting AFC <5-7 was also estimated and compared with FSH.

Results: The nomogram showed that serum levels of inhibin B rapidly decreased after the age of 40. Inhibin B was positively correlated with AMH ($R = 0.57$, $P < 0.001$), AFC ($R = 0.34$, $P < 0.001$) and testosterone ($R = 0.10$, $P = 0.002$), and negatively correlated with FSH ($R = -0.41$, $P < 0.001$) and LH ($R = -0.20$, $P < 0.001$) and FSH/LH ($R=-0.18$, $P < 0.001$), while no correlation was found with PRL. Unexpectedly, Inhibin B (AUC = 0.74, $P < 0.001$ for the establishment population; AUC = 0.78, $P < 0.001$ for the validation population) had a slightly higher value than FSH (AUC = 0.71, $P < 0.001$ for the establishment population; AUC = 0.72, $P < 0.001$ for the validation population) in diagnosing AFC <5-7.

Conclusions: For healthy reproductive age women, the decline of inhibin B can reflect decreased ovarian reserve effectively, having a good consistency with AMH and AFC. More importantly, inhibin B had an advantage in predicting AFC <5-7 compared with FSH, which suggested the potential of inhibin B in predicting ovarian response. These results

will be helpful to the clinical application of inhibin B in the evaluation of female ovarian reserve and the assessment of their reproductive capacity. Trial registration: http://clinicaltrials.gov; NCT02294500.

Keywords: Inhibin B, follicle-stimulating hormone, anti-Mullerian hormone, antral follicle count, ovarian reserve, fertility

INTRODUCTION

Inhibin B, a heterodimeric glycoprotein that comprises an alpha subunit linked to a beta-B subunit, belongs to the superfamily of transforming growth factor-β. Secreted by granulosa cells of developing follicles, the non-steroidal hormone is well known for its property of suppressing follicle-stimulating hormone (FSH). A high level of inhibin B in the serum directly exerts negative feedback on the pituitary gland, leading to a decrease in FSH. Therefore, the higher level of serum inhibin B of reproductive age women is one of the important factors to maintain a low level of serum FSH. However, with the increase of their age, both the quality and quantity of ovarian follicles decrease, the level of serum inhibin B decreases gradually, and the inhibitory effect on FSH will be weakened, which is also one of the important reasons for the progressive increase of their serum FSH levels (1–3).

With continuous study, researchers gradually realized the importance of Inhibin B in female fertility. The findings of previous studies suggested that inhibin B may have certain clinical application potential in assessing the progress of ovarian aging, diagnosing premature ovarian failure (POF) or premature ovarian insufficiency (POI), evaluating the ovarian function of cancer survivors, and predicting assisted reproductive technology (ART) outcomes. Welt et al. found that the decrease in inhibin B was the earliest marker of the decline in follicle number across reproductive aging (4). Bidet et al. found that inhibin B was one of the predictive factors for the resumption of ovarian function in POF patients (5). Recently, Zhu and colleagues revealed that there was a significantly continuous decline in inhibin B accompanying the progress of POI (6). Studies on cancer survivors showed significantly lower inhibin B levels in cancer survivors (7, 8). However, other studies showed no significant difference between cancer survivors and controls (9, 10). Due to differences in the study populations, the inclusion criteria used, and the methodologies used in several laboratories, the conclusions of studies on inhibin B and ovarian response and ART outcomes varied (11–15). Collectively, studies on the clinical application value of inhibin B were still had inconsistent findings, and the evidence was insufficient.

Moreover, few studies have focused on the variation tendency and reference range of inhibin B in healthy reproductive age women, which is necessary to be determined, will contribute to a better assessment of ovarian function and facilitate the clinical application of inhibin B. Despite the potential value of inhibin B, the uniform normative data for female adults are rare worldwide. Besides, the relationship between inhibin B and other classical ovarian reserve markers including FSH, anti-Mullerian hormone (AMH), and antral follicle count (AFC) remains unclear to date. To define the variation trend of inhibin B in female adults with age, and explore its value in the reflection of ovarian reserve and function, we detected the levels of inhibin B in a group of reproductive age women and investigated whether a correlation exists between inhibin B and other important ovarian reserve and function makers including AFC, FSH, AMH, LH, prolactin (PRL), testosterone (T), and progesterone.

METHODS

Study Centers

Since October 2011, a nationwide, standardized, systematic research protocol was used for women over 20 years old in this prospective and open-label study. A group of healthy Chinese females was recruited (n=2524) through advertisements to establish an ovarian reserve database that included clinical and biological factors. Six universities and eight medical institutes participated in this recruitment.

Inclusion Criteria

This research included a questionnaire regarding fertility, family history, and climacteric complaints, as well as ultrasonography and blood examination. Volunteers were enrolled in the study if they met all of our criteria, which were also adopted in our previous studies (16, 17). The inclusion criteria were as follows: (1) for women <40 years old having regular menstrual cycles and for women >40 not required to have regular menstrual cycles considering that they may be in normal perimenopause or menopause; (2) no hormone therapy in the past 6 months; (3) no history of radiotherapy or chemotherapy; (4) no history of hysterectomy, oophorectomy, or any other type of ovarian surgery; (5) no ovarian cysts or ovarian tumors; (6) no known chronic, systemic, metabolic, or endocrine diseases such as hyperandrogenism or hyperprolactinemia.

Ethics and Informed Consent

The clinical investigation followed the Declaration of Helsinki, and the protocol was approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. All eligible patients gave written informed consent before entering this study. The clinical data from all patients came from the ovarian aging database v1.0 (http://clinicaltrials.gov; NCT02294500).

Study Design

According to international standards, a reference range based on at least 120 individuals is the preferred method (18). After

54 women with missing values, 2 women older than 50 years, and 915 women who did not meet inclusion criteria strictly were excluded, the data from 1553 women were left for our study. The data from 948 women were employed to establish the reference range, and the remaining 605 were utilized for further validation.

Each volunteer had a face-to-face interview using a prepared questionnaire. Blood samples from the follicular phase or day 2–5 of the menstrual cycle were drawn from an antecubital vein, clotted, and centrifuged, and the serum was aliquoted sterile and stored at −80°C until hormone analyses were performed. The flowchart of the study was shown in **Figure 1**.

Hormones Assays

Serum levels of inhibin B, AMH, FSH, LH, estradiol (E2), PRL, progesterone, and T were evaluated. Levels of inhibin B and AMH were measured using inhibin B ELISA kits and AMH ELISA kits from Beckman Coulter Inc., which were described in the previous research (19). All serum measurements for the patients were performed in the same laboratory using the same assays. The assay was developed using a sequential application of sample, conjugated antibody, substrate, and amplifier, with the washing as instructed. Absorbance was read in a microplate reader at a wavelength of 450 nm, along with 620 nm used as a reference filter.

Sample concentrations of inhibin B were extrapolated from the standard curve using a cubic-cubic regression. The detection limit of the assay was 2.6 pg/mL, the intra- and inter-assay coefficients of variation were 3.8% and 5.2%, respectively. Samples with inhibin B concentrations less than the detection limit of the assay were assigned a value of 2.5 for analysis.

The levels of FSH, LH, E2, PRL, progesterone, and T were measured using a chemiluminescence-based immunometric assay on an ADVIA Centaur immunoassay system (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA). The manipulation was performed following the manufacturer's instructions.

AFC Evaluation

This ultrasound examination was performed at the multi centers. All participating research institutes were modernized large comprehensive hospitals and had our regular supervision and verification. We formulated the unified standard for this examination in the beginning, and all ultrasound doctors were strictly trained and tested AFCs according to the same standard. AFC was regarded as the total number of visible, round or oval, intra-ovarian transonic follicles with a diameter ranging from 2 to 10 mm. Ultrasound examinations were performed by experienced fertility specialists in each of the participating research institutes. If one or both ovaries could not be spotted, the AFC was defined as not visible.

Statistical Analysis

Shapiro-Wilk test was conducted to test the distribution types of continuous variables and found that they all conformed to

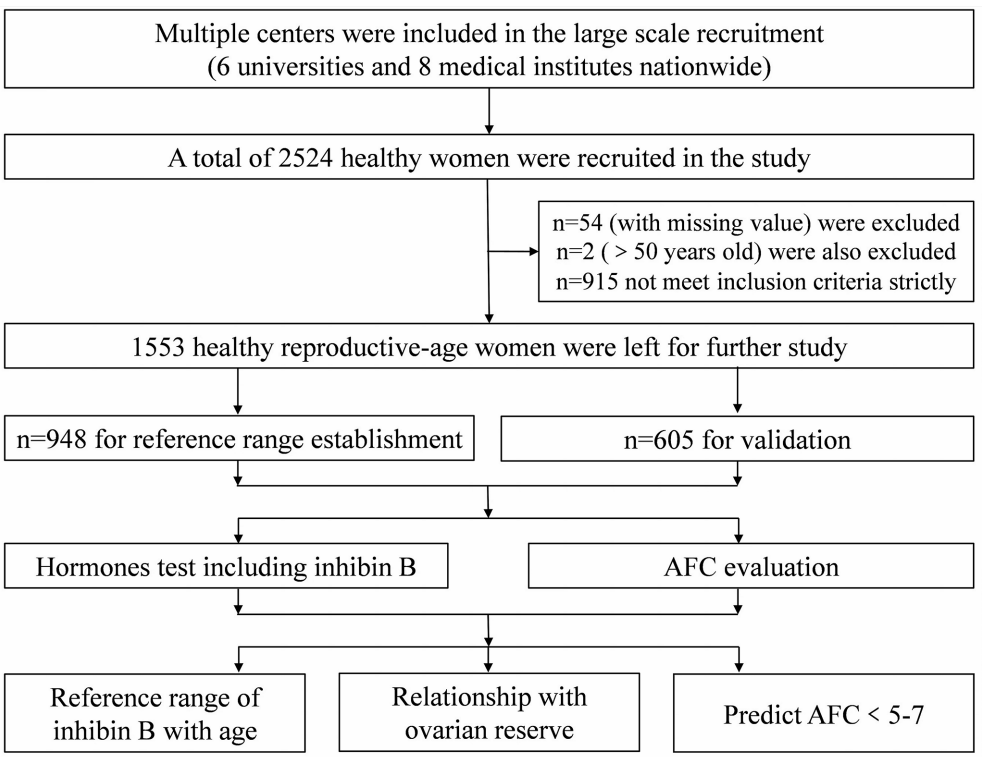

FIGURE 1 | The flowchart of this study.

skewed distributions. Therefore, they were presented as median and 90% prediction interval (5-95 percentiles). Variables, such as inhibin B and AMH, were also logarithmically transformed in the case of significant deviation from the normal distribution. The reference ranges were illustrated according to the value of 90th percentile, median and 10th percentile, as well as mean+2 SD and mean−2 SD, which has also been reported by a previous study (20). Inhibin B levels across different age groups (5-year intervals before 40 years old or 10-year intervals after 40 years old) in adult women were analyzed by the Kruskal-Wallis test, and the value for each group (each age interval) was compared with those of the previous group using the Mann-Whitney U test, which was also adopted in our previous study (16). Spearman correlation analyses were used in our study to calculate the relationships between inhibin B levels and age, BMI, as well as other ovarian reserve or function markers. As a novel method that has also been employed by other scholars (21), nomogram curves for the distribution of the inhibin B levels as a function of age were also calculated. All *P*-values were two-tailed, and values < 0.05 were considered statistically significant. Receiver operating characteristic (ROC) curves were plotted to evaluate the value of inhibin B and FSH in predicting AFC <5-7. All statistical analyses were carried out using the IBM SPSS Statistics 13.0 statistical software package (SPSS Inc., Chicago, IL, USA).

RESULTS

The Basal Clinical Characteristics

The median age of the establishment population was 32.3 years (range 5%-95%, 22.7–44.4 years), and 28.7 years (range 5%-95%, 23.9-34.3 years) for the validation population. AFC, serum hormones, including inhibin B, FSH, LH, PRL, progesterone, E2, T, and AMH were measurable in the majority of individuals (**Table 1**).

The Variation Trend of Inhibin B in Healthy Women of All Ages

It can be deduced that the level of inhibin B rapidly decreases after the age of 40, based on the nomogram of the cubic regression model (**Table 1**, **Figure 2A**). We chose this model because it had the least sum of squared residuals compared with the linear regression model and the quadratic regression model. According to both the highest R^2 and the convenience of interpretation, the cubic model was observed to be the best of these regression models and was chosen as the most appropriate one to illustrate the relationship between inhibin B and age. The confidence interval (CI) for 5%, 10%, 25%, 50%, 75%, 90% and 95% was also depicted in this nomogram.

The reference range of inhibin B for each age group was expressed as mean and median (**Table 1**, **Supplementary Tables 1, 2**). For women 20-25, 25-30, 30-35, 35-40, and ≥ 40 years old,

TABLE 1 | Median and 90% prediction interval (5–95 percentiles) of serum hormones and AFC in different age groups among the establishment population (n=948).

	Age groups					
	<25y	**25–30y**	**30–35y**	**35–40y**	**> 40y**	**All**
Number	200	200	200	202	146	948
Age(y)	23.7	27.6	32.3	37.3	42.5	32.3
5–95 percentiles	21.1-24.9	25.3-29.7	30.3-34.6	35.2-39.8	40.2-48.7	22.7-44.4
BMI (kg/m^2)	19.8	20.4	20.9	22.1	23.0	21.3
5–95 percentiles	16.4-25.4	17.0-27.0	17.7-27.5	18.1-27.4	19.5-27.8	17.5-27.3
Inhibin B (pg/mL)	82.6	79.8	88.0	74.5	55.1	78.1
5–95 percentiles	26.8-137.1	39.8-160.4	35.6-139.1	27.8-127.1	2.6-135.5	12.1-137.4
$_{log}$inhibin B (pg/mL)	4.4	4.4	4.5	4.3	4.0	4.4
5–95 percentiles	3.3-4.9	3.7-5.1	3.6-4.9	3.3-4.9	0.9-5.0	2.5-4.9
FSH (mIU/mL)	6.1	6.8	6.9	7.1	7.9	7.0
5–95 percentiles	3.8-9.5	4.3-10.5	4.6-10.0	4.8-11.2	4.6-26.4	4.4-12.5
LH (mIU/mL)	4.6	4.4	3.9	3.8	4.5	4.2
5–95 percentiles	2.1-10.4	2.2-9.9	1.8-8.6	1.7-8.9	1.7-12.1	1.9-10.0
Estradiol (pg/ml)	39.6	40.3	43.0	42.4	40.6	41.2
5–95 percentiles	21.5-75.3	16.9-91.7	15.8-89.0	14.0-91.0	12.3-203.0	16.6-96.1
AMH (ng/ml)	6.2	5.6	4.2	3.2	1.0	3.7
5–95 percentiles	2.1-12.7	1.6-13.3	1.4-11.6	0.6-9.5	0.1-5.5	0.1-11.7
$_{log}$AMH (ng/ml)	0.8	0.7	0.6	0.5	0.01	0.6
5–95 percentiles	0.3-1.1	0.2-1.1	0.2-1.1	-0.2-1.0	-1.1-0.7	-1.1-1.1
PRL (ng/ml)	13.4	14.0	13.0	11.1	10.6	12.2
5–95 percentiles	7.1-39.7	7.3-29.9	6.3-32.1	5.7-26.6	5.3-28.4	6.2-30.2
PRG (ng/ml)	0.6	0.6	0.6	0.5	0.5	0.6
5–95 percentiles	0.1-1.3	0-1.5	0-1.2	0.1-1.0	0.2-1.3	0.1-1.2
T (ng/dl)	32.5	30.3	30.0	25.0	18.0	26.6
5–95 percentiles	9.9-61.1	9.0-60.9	6.5-58.7	2.5-46.2	0-41.7	2.5-55.9
AFCs	14	13	11	10	4	11
5–95 percentiles	8-24	5-22	5-20	2-18	0-14	2-21

BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; AMH, anti-Mullerian hormone; PRL, prolactin; PRG, progesterone; T, testosterone; AFC, antral follicle counts; y, year.

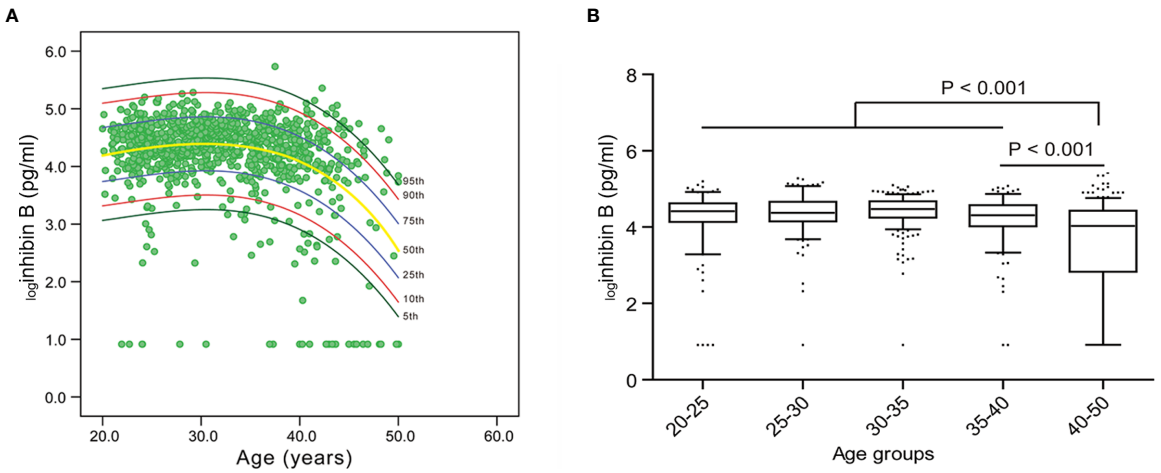

FIGURE 2 | Nomogram for inhibin B and the variation trend of inhibin B with age. **(A)** Inhibin B nomogram based on the cubic regression model. Each line represents the value of confidential interval (CI). A rapid decrease of inhibin B value was observed in women after 40 years old. **(B)** Median value of inhibin B in each age group.

the median value of inhibin B was 82.6 pg/ml, 79.8 pg/ml, 88.0 pg/ml, 74.5 pg/ml, and 55.1 pg/ml, respectively. The level of $_{log}$inhibin B of 40-50 years old women was significantly lower than 35-40 years old women (mean 3.6 vs 4.3, median 4.03 *vs* 4.31, P<0.001). Furthermore, the value between the age group of 20-40 years old and 40-50 years old was also significantly different (mean 4.3 *vs* 3.6, median 4.41 *vs* 4.03, P<0.001) (**Figure 2B**).

Relationship Between Inhibin B and Hormones Secreted by the Pituitary Gland

We evaluated the relationship between inhibin B and FSH, LH, and PRL. Inhibin B showed a significant negative correlation to FSH (R = -0.41, P < 0.001) and LH (R = -0.20, P < 0.001), while no correlation was found between Inhibin B and PRL (**Figures 3A, B, D**). We also analyzed the correlation between these hormones and age and found a significant positive correlation between FSH (R=0.30, P< 0.001) and age, while PRL (R=-0.17, P< 0.001) was negatively correlated with age, and no correlation was found between LH and age (**Supplementary Figure 1**). The previous study has found that FSH/LH ratio can forecast poor and excessive ovarian response in IVF-ICSI (22), so we also analyzed this variable, and found that FSH/LH was significantly negatively correlated with inhibin B (R=-0.18, P < 0.001), and was positively correlated with age (R=0.27, P< 0.001) (**Figure 3C**, **Supplementary Figure 1**).

Relationship Between Inhibin B and AFC, Hormones Secreted by the Ovary

We evaluated the relationship between inhibin B and AMH, AFC, progesterone, and T. AMH and AFC are classic markers for ovarian reserve, while progesterone and T are hormones secreted by ovaries, which can also reflect the ovarian function. Inhibin B was positively correlated with $_{log}$AMH (R = 0.57, P < 0.001), AFC (R = 0.34, P < 0.001) and, T (R = 0.10, P = 0.002), while the association between inhibin B and progesterone was not significant (**Figure 4**). We also analyzed the relationship between these variables and age and found that $_{log}$AMH (R=-0.54, P< 0.001), AFC (R=-0.52, P< 0.001), progesterone (R=-0.09, P=0.01) and T (R=-0.31, P< 0.001) were all significantly negatively correlated with age (**Supplementary Figure 1**).

Inhibin B Showed Greater Potency in Predicting AFC<5-7 Compared to FSH

Because FSH showed a significant negative correlation with inhibin B, a nomogram was also built based on the cubic regression model. It can also be deduced that FSH rapidly increases after approximately 40 years of age (**Figure 5A**). The cubic model was chosen as the most appropriate model to illustrate the relationship between FSH and age because it had both the highest R^2 and the least sum of squared residuals and is convenient to interpret compared with the linear regression model and quadratic regression model. The confidence interval (CI) for 5%, 10%, 25%, 50%, 75%, 90% and 95% are also depicted in this nomogram. It can be speculated that there are more women over 40 years old with low levels of inhibin B and high levels of FSH.

According to the 'Bologna' criteria, AFC less than 5-7 follicles is one of the diagnosis criteria for poor ovarian response (23). Therefore, we indirectly compared the ability of inhibin B and FSH to predict ovarian response by comparing their predictive ability to AFC < 5-7. The data in the establishment group and validation group were employed to calculate the area under the ROC curve (AUC). Inhibin B (AUC = 0.74, P < 0.001 for the establishment population; AUC = 0.78, P < 0.001 for the validation population) had a slightly higher value than FSH (AUC = 0.71, P < 0.001 for the establishment population; AUC = 0.72, P < 0.001 for the validation population) in predicting AFC < 5-7 (**Figure 5B**).

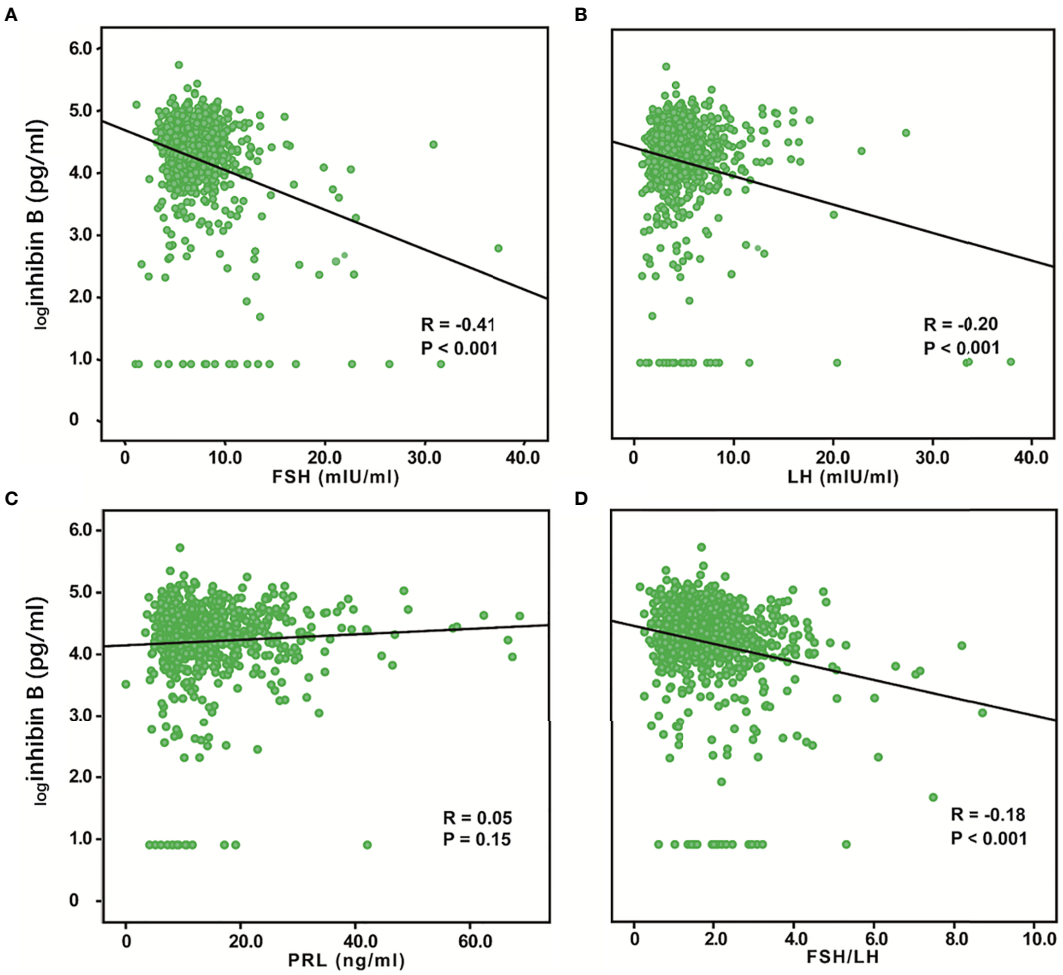

FIGURE 3 | Relationship between serum inhibin B levels and FSH, LH, PRL, and FSH/LH. $_{log}$Inhibin B was significantly negatively correlated with FSH (R = -0.41, P < 0.001) **(A)** and LH (R = -0.20, P < 0.001) **(B)**. No correlation was found between logInhibin B and PRL (R = 0.05, P =0.15) **(C)**. $_{log}$Inhibin B was significantly negatively correlated with FSH/LH (R = -0.18, P < 0.001) **(D)**.

DISCUSSION

The variation trend of inhibin B was detected and a normal reference range was successfully established in healthy reproductive age Chinese women, which was also validated in a group of women less than 40 years old. We found that inhibin B levels were detectable in 95% of the individuals, and the levels were nearly the same among women 20-35 years old, but significantly decreased in adult women over 40 years old. This finding is consistent with the previous finding that women's ovarian reserve and fertility decreased at a drastic speed after approximately 37.5 years (24).

Although the reference range of inhibin B remains unclear, the data is still rare, inhibin B has already been widely studied for its important role in the regulation of the hypothalamus-pituitary-gonadal axis through suppressive effects on activin-mediated FSH expression and release, and the direct effects on ovarian folliculogenesis, steroidogenesis and menstrual cycle, which can affect AFC results (25). FSH and AFC are traditional ovarian reserve indicators, therefore, inhibin B may have a potential role in reflecting ovarian reserve too. Our results on the normal reference range of inhibin B may contribute to a more accurate evaluation of ovarian reserve. Besides, FSH is an indirect marker of ovarian reserve and influenced by hypothalamic function, ovarian factors, and steroid hormones. In contrast, the inhibin B concentration would be a more direct marker of the ovarian reserve because it is produced by small ovarian follicles and is therefore direct measures of the follicular pool (26, 27).

In addition to finding that the inhibin B levels of reproductive age women decreased with age, our study also revealed that serum inhibin B levels were significantly positively correlated with AMH and AFC, and negatively correlated with age, FSH, LH, and FSH/LH. These results were in accord with the previous studies (4, 18, 28, 29) (**Supplementary Tables 3, 4**). Although some research conclusions were still controversial, most studies

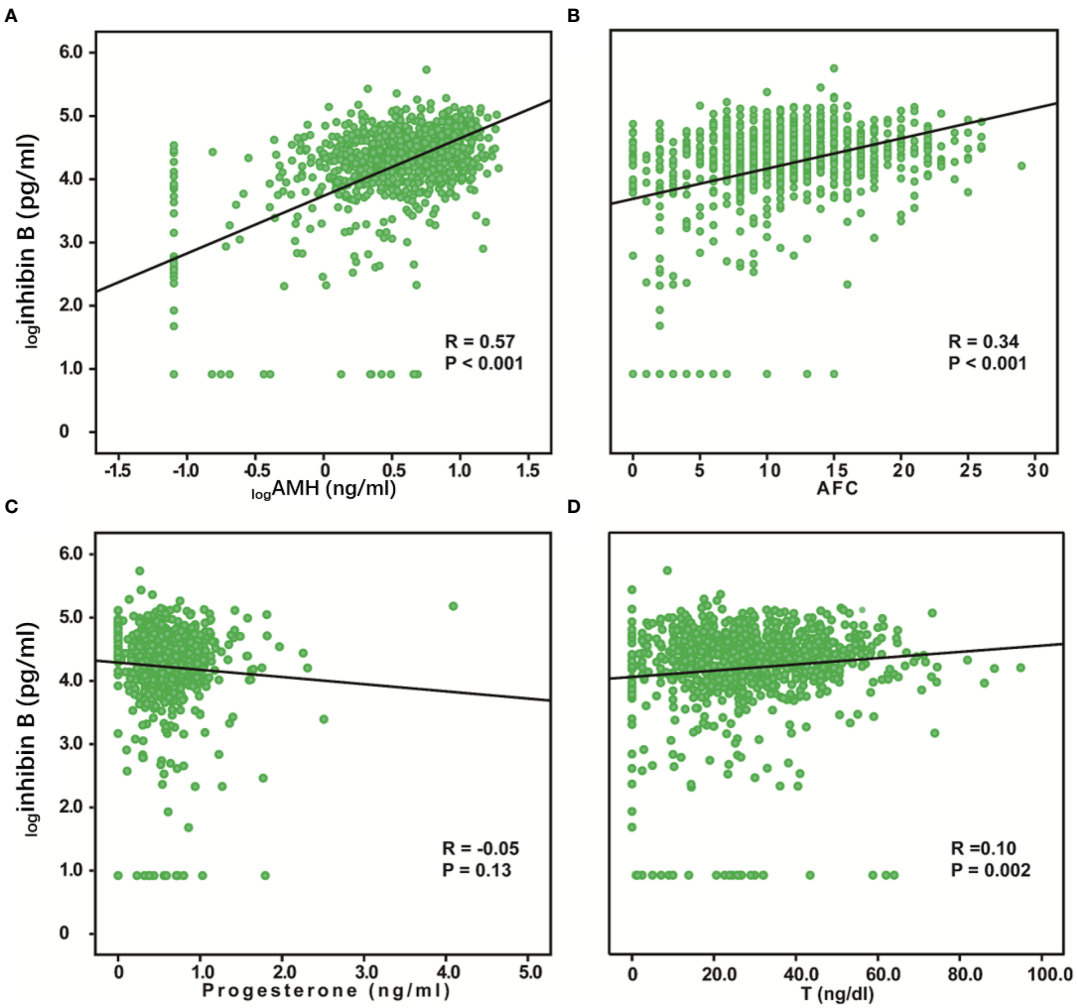

FIGURE 4 | Relationship between serum inhibin B levels and AMH, AFC, Progesterone, and T. $_{log}$Inhibin B was significantly positively correlated with $_{log}$AMH (R = 0.57, P < 0.001) **(A)**, AFC (R =0.34, P < 0.001) **(B)**, and T (R = 0.10, P =0.002) **(D)**. No correlation was found between $_{log}$Inhibin B and progesterone (R = -0.05, P =0.13) **(C)**.

on women with impaired ovarian function, including POF or POI patients (5, 6, 30–32), diminished ovarian reserve (DOR) patients (33, 34), and cancer survivors (7, 8), also found a dramatic decrease in inhibin B, most of them were below the detection limit (**Supplementary Table 5**). These pieces of evidence further indicate that inhibin B has a certain potential in evaluating ovarian reserve.

However, inhibin B is currently not a reliable measure of ovarian reserve in clinical practice. The main reasons may be as follows. Firstly, Inhibin B concentrations fluctuate with the menstrual cycle and ART cycle (35, 36) (**Supplementary Table 3**). The previous study has found that the concentration of inhibin B rose rapidly in the early follicular phase to a peak on the day after the intercycle FSH rise, then fell progressively during the remainder of the follicular phase, two days after the midcycle LH peak, there was a short-lived peak in the inhibin B concentration, which then fell to a low concentration for the remainder of the luteal phase (35). Secondly, due to different populations and detection kits in different studies, the absolute values of inhibin B concentrations were also different. Thus, clinicians may find it difficult to generalize inhibin B cut-off values in the medical literature to clinical practices unless they are using the very same assay and reference preparation. Further efforts are needed to standardize the detection technology of inhibin B. Thirdly, limited by small sizes, heterogeneity among study design, analyses and outcomes, and the lack of validated outcome measures, the ability of inhibin B to assess ovarian reserve is still controversial. More prospective studies with larger sample sizes are needed to provide more reliable evidence.

Despite recent striking advances in ART, poor ovarian reserve diagnosis and treatment is still considered challenging. The core of the pathophysiology of poor ovarian response is the limited number of follicles responding to FSH (37). Because our subjects had not undergone ART, we were not able to directly evaluate the

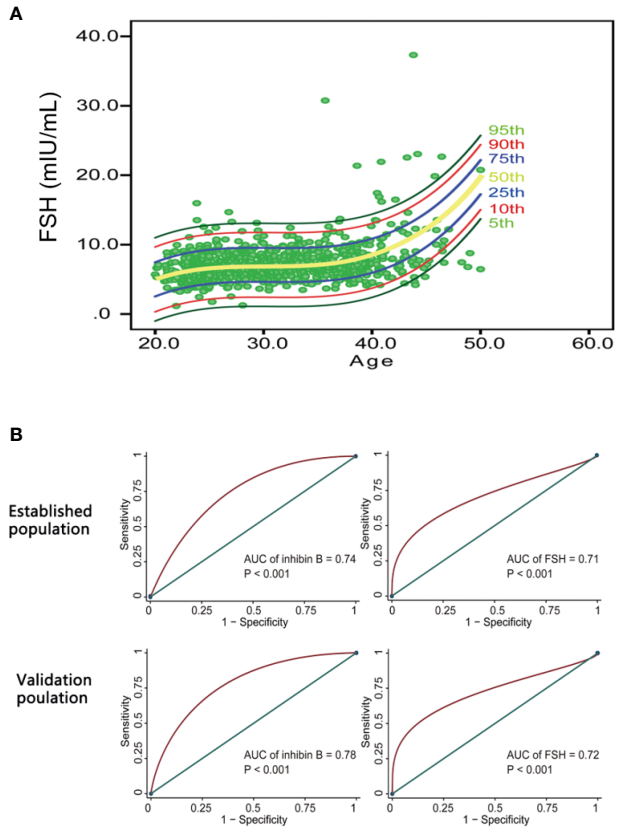

FIGURE 5 | The potency of inhibin B and FSH in predicting AFC<5-7. **(A)** FSH nomogram based on cubic regression model. Each line represents the value of confidential interval (CI). Rapid increase of FSH value was observed in women after 40 years old. **(B)** ROC curves evaluating the diagnostic value of inhibin B and FSH in predicting AFC < 5-7. Inhibin B (AUC = 0.74, P < 0.001 for the establishment population; AUC = 0.78, P < 0.001 for the validation population) had a slightly higher value than FSH (AUC = 0.71, P < 0.001 for the establishment population; AUC = 0.72, P < 0.001 for the validation population) in diagnosing AFC<5-7.

response of their ovaries to controlled superovulation. Therefore, we changed our thinking and indirectly compared the ability of inhibin B and FSH to predict ovarian response by comparing their predictive ability to AFC < 5-7, which is one of the diagnostic criteria of poor ovarian response (23). In the end, we found that inhibin B had a slight advantage over FSH in predicting AFC < 5-7, suggesting that inhibin B may also have a role in evaluating ovarian response in women. Some previous studies also supported the value of inhibin B in predicting ovarian response and ART outcomes (11, 14, 15, 38–47), but the conclusions were still controversial (12, 13, 48, 49) (**Supplementary Table 6**). Moreover, a recent study on older reproductive age women found no association between inhibin B and reduced fertility (50). Collectively, more studies are needed, especially prospective studies with large sample sizes, to further clarify the relationship between inhibin B and natural fertility and ART outcomes.

This study had several strengths. The first one was the study design, which was a multi-center study with large sample size. We recruited a group of healthy reproductive age Chinese women through advertisements to establish an ovarian reserve database that included clinical and biological factors. Volunteers came from a nationwide region including six universities and eight medical institutes. Secondly, by analyzing and comparing the outcome data of 1553 health women in different age groups, we successfully established a reference range for inhibin B and established different levels of correlations among different ovarian reserve markers, explore the potency of inhibin B in evaluating ovarian reserve and ovarian response. Thirdly, updated ELISA kits were applied in our study. The new kits had new standards for measurement and had been renewed with higher accuracy and sensitivity. Therefore, samples that were undetectable in the past can now be detected with the new kits. However, the limitations of the current study deserve careful consideration. Firstly, our study only included healthy reproductive age women, while prepubescent, adolescent girls, and unhealthy adult women were not included. Secondly, we only tested the levels of serum inhibin B in the follicular phase, and there was no information for the variability of inhibin B within a menstrual cycle. Thirdly, there was a lack of data on fertility and ART outcomes, so it was impossible to directly assess the predictive value of inhibin B on fertility and ART outcomes.

CONCLUSIONS

A total of 2,524 healthy reproductive age women from six universities and eight medical institutes participated in this recruitment. The reference range for serum inhibin B was established and validated among healthy adult women. For healthy reproductive age women, the decline of serum inhibin B can reflect decreased ovarian reserve effectively, having a good consistency with AMH and AFC. More importantly, inhibin B had a slight advantage in predicting AFC < 5-7 compared with FSH, which suggested the potential of inhibin B in predicting ovarian response. These results will be helpful to the clinical application of inhibin B in the evaluation of female ovarian reserve and the assessment of their reproductive capacity.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology. The patients/participants provided their written informed consent to participate in this study. The patients/

participants provided their written informed consent for potentially identifiable human images or data presented in this study.

AUTHOR CONTRIBUTIONS

JW and JZ contributed to conceptualization, data curation, methodology, supervision, and writing the original draft. XD, HZ, TD, CZ, WM, YZ, WQ, YL, ZL, SD, YJ and AL contributed to healthy population recruitment and data collection. KH contributed to formal analysis, methodology, conceptualization, data curation, and writing the original draft. SW contributed to conceptualization, data curation, methodology, and supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.626534/full#supplementary-material

Supplementary Figure 1 | The levels of hormones and BMI changed with age. $_{log}$AMH (R = -0.54, P < 0.001) **(A)**, AFC (R =-0.52, P < 0.001) **(C)**, progesterone (R = -0.09, P =0.01) **(G)**, PRL (R = -0.17, P < 0.001) **(H)**, and T (R = -0.31, P < 0.001) **(I)** were significantly negatively correlated with age. FSH (R = 0.30, P < 0.001) **(B)**, BMI (R = 0.32, P < 0.001) **(D)**, and FSH/LH (R = 0.27, P < 0.001) **(E)** were significantly positively correlated with age. No correlation was found between LH (R = 0.03, P =0.41) **(F)** and age.

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Melatonin: Multi-Target Mechanism Against Diminished Ovarian Reserve Based on Network Pharmacology

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Background: Diminished ovarian reserve (DOR) significantly increases the risk of female infertility and contributes to reproductive technology failure. Recently, the role of melatonin in improving ovarian reserve (OR) has attracted widespread attention. However, details on the pharmacological targets and mechanisms of melatonin-improved OR remain unclear.

Objective: A systems pharmacology strategy was proposed to elucidate the potential therapeutic mechanism of melatonin on DOR at the molecular, pathway, and network levels.

Methods: The systems pharmacological approach consisted of target identification, data integration, network construction, bioinformatics analysis, and molecular docking.

Results: From the molecular perspective, 26 potential therapeutic targets were identified. They participate in biological processes related to DOR development, such as reproductive structure development, epithelial cell proliferation, extrinsic apoptotic signaling pathway, PI3K signaling, among others. Eight hub targets (MAPK1, AKT1, EGFR, HRAS, SRC, ESR1, AR, and ALB) were identified. From the pathway level, 17 significant pathways, including the PI3K-Akt signaling pathway and the estrogen signaling pathway, were identified. In addition, the 17 signaling pathways interacted with the 26 potential therapeutic targets to form 4 functional modules. From the network point of view, by regulating five target subnetworks (aging, cell growth and death, development and regeneration, endocrine and immune systems), melatonin could exhibit anti-aging, anti-apoptosis, endocrine, and immune system regulation effects. The molecular docking results showed that melatonin bound well to all hub targets.

Conclusion: This study systematically and intuitively illustrated the possible pharmacological mechanisms of OR improvement by melatonin through anti-aging, anti-apoptosis, endocrine, and immune system regulation effects.

Keywords: diminished ovarian reserve (DOR), ovarian reserve (OR), melatonin, potential therapeutic targets, signaling pathways, biological processes, network pharmacology

INTRODUCTION

Infertility affects a significant proportion of humanity and is regarded as a global public health issue by the World Health Organization (1, 2). Diminished ovarian reserve (DOR), defined as a reduction in both oocyte quality and quantity, is one of the most common causes of female infertility and poor ovarian response to controlled ovarian stimulation with a rapidly increasing occurrence rate (3, 4). In addition, women with DOR have exceedingly high rates of recurrent pregnancy loss and no euploid embryos (5–7). Devine et al. reported that the prevalence of DOR increased from 19 to 26% in the past few years, representing a major challenge in reproductive medicine (8, 9). Despite its prevalence, its pathology remains unclear. Aging is the most common cause of DOR. Other influential factors for DOR include genetic predisposition, autoimmune diseases, chemotherapy, and psychological stress (10–13).

The decline of ovarian reserve (OR) is a continuous, gradual process starting from the oocyte death of embryos at 20 weeks of gestation until menopause (14). The premature depletion of OR eventually results in premature ovarian failure, a more severe condition, which might lead to a loss of reproductive capacity, seriously affecting women's quality of life (15, 16). Thus, early and active interventions should be implemented in women with DOR before it is too late. However, DOR treatment remains a significant challenge in reproductive medicine, although various treatment strategies are currently being used (9). For example, DHEA, as an adjuvant therapy in *in vitro* fertilization (IVF), might increase the number of retrieved oocytes (17); however, the true benefit is under active debate as DHEA has some side effects, including acne, sleep problems, and headaches (18).

Melatonin (5-methoxy-N-acetyl tryptamine), a pineal gland hormone, plays a significant role in regulating the circadian sleep-wake cycle, reproductive physiology, and immune functions (19). As a dietary supplement, it has gained widespread popularity globally. Lerner and colleagues' discovery of melatonin in 1958 presented a new research avenue in reproductive physiology (20, 21). Since Wurtman et al. reported that preovulatory follicles contain substantial amounts of melatonin, which may affect ovarian steroidogenesis, many studies have focused on the role of melatonin in OR (21). Morioka et al. conducted the first clinical trial to evaluate melatonin as a drug for improving oocyte quality in women who could not become pregnant because of poor-quality oocytes (22). The results showed that melatonin treatment increased oocyte quality. Interestingly, the melatonin-treated group's intrafollicular melatonin concentration was four times higher than that of the control group, consistent with Morioka's study. Similarly, several subsequent studies have confirmed that melatonin supplementation can ameliorate intrafollicular oxidative balance, improve the quantity and quality of oocytes, and improve IVF outcomes in women with DOR and infertility (23–27). Some experts have suggested that melatonin levels in the follicular fluid may serve as a biomarker for predicting OR (28, 29). In addition, animal experiments have also confirmed that melatonin can protect the quality of oocytes and improve OR through multiple mechanisms (30–35).

Although anti-DOR activities exerted by melatonin have been reported, in-depth mechanistic preclinical studies are currently limited. Moreover, details of biomarkers and the biological pathways through which melatonin exerts its effects in improving OR are yet to be completely elucidated. In a previous study, a network pharmacology-based approach was successfully used to uncover the target proteins and potential therapeutic mechanisms of drugs (36–38). Accordingly, this study was performed to reveal the predictive targets and therapeutic mechanisms underlying melatonin action against DOR using a systematic network pharmacology-based approach. **Figure 1** illustrates the workflow of the study.

Abbreviations: AKT1, RAC-alpha serine/threonine-protein kinase; ALB, Albumin; AR, Androgen receptor; ART, Assisted reproductive technology; BC, Betweenness centrality; BP, Biological process; CC, Closeness centrality; DC, Degree centrality; DOR, Diminished ovarian reserve; EGFR, Epidermal growth factor receptor; ESR1, Estrogen receptor; GO, Gene Ontology; HRAS, GTPase HRas; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK1, Mitogen-activated protein kinase 1; OR, Ovarian reserve; PPI, Protein–protein interaction; SMILES, Simplified molecular input entry specification; SRC, Proto-oncogene tyrosine-protein kinase Src; STRING, The Search Tool for the Retrieval of Interacting Genes; TCMSP, Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; Uniprot, Universal Protein.

MATERIALS AND METHODS

Identification of Putative Melatonin Targets

The PubChem database (https://pubchem.ncbi.nlm.nih.gov/) was used to obtain simplified molecular-input line-entry specification (SMILES) information and the 3D structure of melatonin (39). Melatonin's 3D structure was uploaded to the PharmMapper Server (http://www.lilab-ecust.cn/pharmmapper/), and the SMILES for melatonin was uploaded to the SwissTargetPrediction database (http://www.swisstargetprediction.ch/) to predict the potential melatonin targets (40, 41). DrugBank (https://go.drugbank.com/), SuperTarget (http://insilico.charite.de/supertarget/index.php), and TCMSP (https://tcmspw.com/tcmsp.php) databases were used to identify known melatonin targets (42, 43). All retrieved target names were corrected to official symbols using the UniProt database(https://www.uniprot.org/).

Selection of DOR-Associated Targets

DisGeNET (https://www.disgenet.org/), GeneCards (https://www.genecards.org/), and NCBI Gene databases (https://www.ncbi.nlm.nih.gov/gene/) were utilized to identify targets related to DOR (44). The keyword was "diminished ovarian reserve." To enhance the credibility of the results, DOR-related targets with a gene-disease score ≥0.1 were set in DisGeNET, and the threshold of relevance score was set at 10 in GeneCards.

Protein-Protein Interaction (PPI) Data

The protein-protein interaction data were integrated and obtained from the Search Tool for the Retrieval of Interacting Genes (STRING) platform (https://string-db.org/) (45). The species was limited to "Homo sapiens," and the interaction confidence score was set at 0.7, defined as high confidence on the STRING platform.

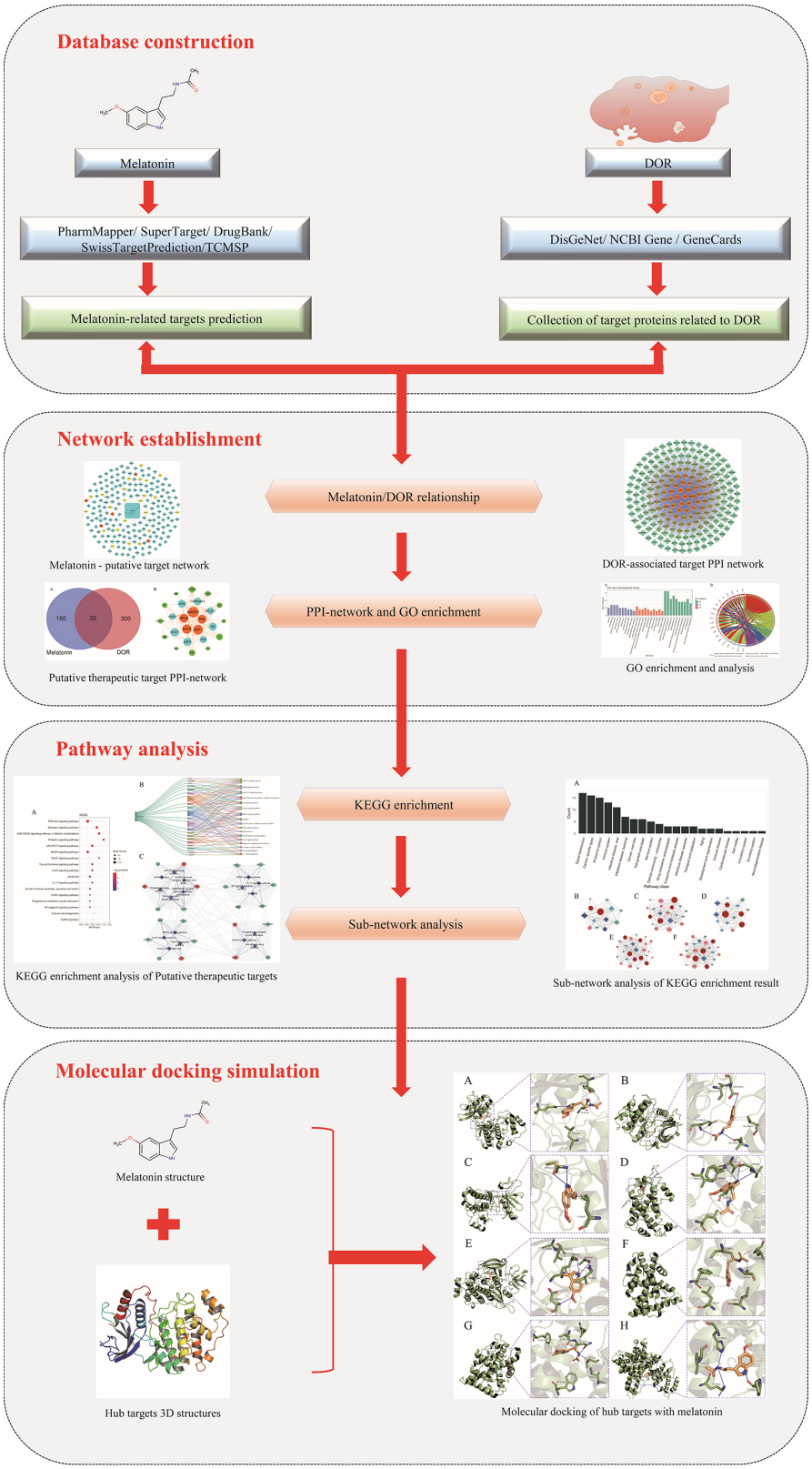

FIGURE 1 | Research workflow diagram.

GO and KEGG Pathway Enrichment

To clarify the role of potential therapeutic targets in gene function and signaling pathways, the ClusterProfiler package of R 4.0.2 was used to perform GO and KEGG pathway enrichment analysis of the common genes for melatonin and DOR (46). The pathway class of every KEGG pathway was obtained from the KEGG PATHWAY database (https://www.kegg.jp/kegg/pathway.html) for further analysis.

Network Construction

Five networks were constructed: (1) the melatonin-putative target network was built by connecting melatonin and its targets; (2) the PPI network of DOR targets; (3) another PPI network was constructed using the intersected melatonin and DOR genes; (4) the melatonin-targets-pathways network was established by linking melatonin, its targets, and key pathways with literature support for DOR treatment. For further analysis, the network was divided into functional modules using the Community Cluster algorithm (Glay) of clustermaker2 (47) and (5) the sub-networks of the potential therapeutic targets that were enriched in different key pathway classes were constructed. All of the above networks were established using Cytoscape 3.8.0.

Molecular Docking Simulation

Target Protein Preparation

The crystal structures of the protein receptors were obtained from the RCSB Protein Data Bank (http://www.rcsb.org/). The downloaded protein structures were pretreated with PyMol 2.4.0 to remove the original ligand, solvent molecules, redundant protein chains and add polar hydrogen. Then, AutoDock Tools 1.5.6 was used to compute the Gasteiger and determine the docking box's center and size (48).

Ligand Preparation

The 3D structure of melatonin was treated by polarity hydrogenation and energy minimization using the MMFF94s force field.

Molecular Docking

AutoDock Vina was then used to evaluate melatonin binding and the hub targets by molecular docking (49). Prior to molecular docking, all protein and melatonin structures were converted to PDBQT format using AutoDock Tools 1.5.6. Melatonin was then docked onto the proteins using AutoDock Vina. Finally, the binding affinity calculated by AutoDock Vina was tallied, and the docking result was visualized using PyMol 2.4.0 software (Open-source version).

RESULTS

Melatonin–Putative Target Network

A total of 206 melatonin targets were obtained after removing duplications from the PharmMapper, SuperTarget, DrugBank, SwissTargetPrediction, and TCMSP databases (**Supplementary 1**). Then, the melatonin-target network was constructed using Cytoscape 3.8.0 (**Figure 2**). As shown in **Figure 2**, there were 28 known targets, accounting for 13.6% of the total targets and 183 putative targets, accounting for 88.8% of the total targets. Furthermore, there were five intersecting targets between the potential and known targets.

PPI Network of DOR Targets

A total of 326 DOR-related targets were obtained from the DisGeNET, GeneCards, and NCBI Gene databases (**Supplementary 2**). A PPI network was constructed to demonstrate the interaction of DOR-related targets (**Figure 3**). Forty-five significant DOR-related targets were obtained according to the mean values for degree centrality (DC), betweenness centrality (BC), and closeness centrality (CC), which were 47.14553991, 0.004491643, and 0.527211414, respectively (**Supplementary 3**).

PPI Network of the Potential Therapeutic Targets

Based on the above results, 26 common melatonin and DOR targets (potential therapeutic targets) were obtained using the Venn Diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/) (**Figure 4A** and **Supplementary 4**). Then, the PPI network of these 26 common targets was constructed (**Figure 4B**). To find the hub targets in this complex biological network, the topological parameters were analyzed. As a result, there are eight hub targets in this PPI network according to DC, BC, and CC mean values, including MAPK1, AKT1, EGFR, HRAS, SRC, ESR1, AR, and ALB (**Supplementary 5**). Meanwhile, as shown in **Table 1**, all eight hub targets were significant DOR-related targets. Therefore, these hub targets might play an essential role in OR improvement via melatonin and were used for the subsequent molecular docking study.

GO and KEGG Enrichment Analysis

GO Enrichment Analysis

The 26 potential therapeutic targets were analyzed using the ClusterProfiler package of R 4.0.2. The top 10 terms of each part of the GO enrichment results were selected based on the counts of hit genes and the *p*-value. The results were visualized using the R package's ggplot2 and are shown in **Figure 5A**. After data screening, the top five enriched GO terms of biological processes (BP) are shown in **Figure 5B**. We could clearly identify that the top five enriched BPs of melatonin against DOR effects were mechanistically linked to reproductive structure development, epithelial cell proliferation, the extrinsic apoptotic signaling pathway, phosphatidylinositol 3-kinase signaling, and response to steroid hormones. Seven of the eight hub genes were also enriched in the top five enriched BPs, including MAPK1, AKT1, EGFR, SRC, HRAS, ESR1, and AR.

KEGG Enrichment Analysis of the Potential Therapeutic Targets

We carried out KEGG pathway enrichment analysis of the 26 therapeutic targets using the ClusterProfiler package of R 4.0.2 and obtained 123 pathways with a p-value <0.05. After data screening, 17 significant pathways were identified (**Figure 6A** and **Table 1**). Then, the genes enriched in each pathway were sorted, and a melatonin-target-pathway network was constructed (**Figure 6B**). In the network, the PI3K-Akt signaling pathway (hsa04151) and estrogen signaling pathway (hsa04915) were significantly enriched (**Figure 7**). To fully understand the mechanism of melatonin in

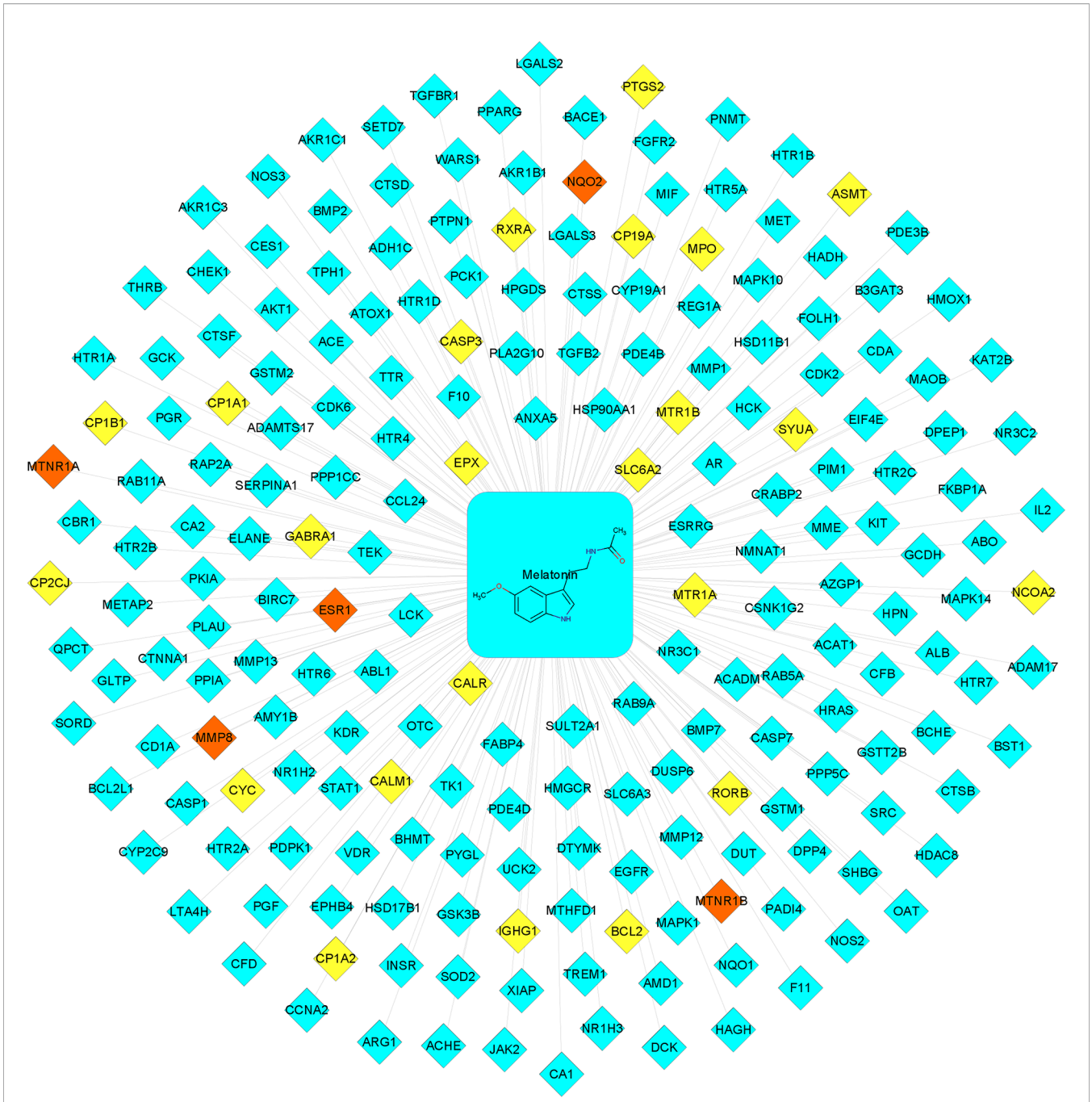

FIGURE 2 | Melatonin–putative target network. The blue-colored nodes represent the potential targets. The yellow-colored nodes represent the known targets. The red-colored nodes represent the intersection of the potential and known targets.

treating DOR, the target-pathway network was decomposed into functional modules using the community cluster (Glay) algorithm of clustermaker2. As illustrated in **Figure 6C**, the target pathway network was divided into four modules. Module 1 contained six pathways, including the AGE-RAGE signaling pathway in diabetic complications (hsa04933), JAK-STAT signaling pathway (hsa04630), thyroid hormone signaling pathway (hsa04919), growth hormone synthesis, secretion, and action (hsa04935), progesterone-mediated oocyte maturation (hsa04914), and GnRH secretion (hsa04929). Module 2 consisted of four pathways, including the VEGF signaling pathway (hsa04370), apoptosis (hsa04210), IL-17 signaling pathway (hsa04657), and NF-κB signaling pathway (hsa04064). Module 3 comprised four pathways, including the PI3K-Akt signaling pathway (hsa04151), MAPK signaling pathway (hsa04010), FoxO signaling pathway (hsa04068), and ovarian steroidogenesis (hsa04913). Module 4

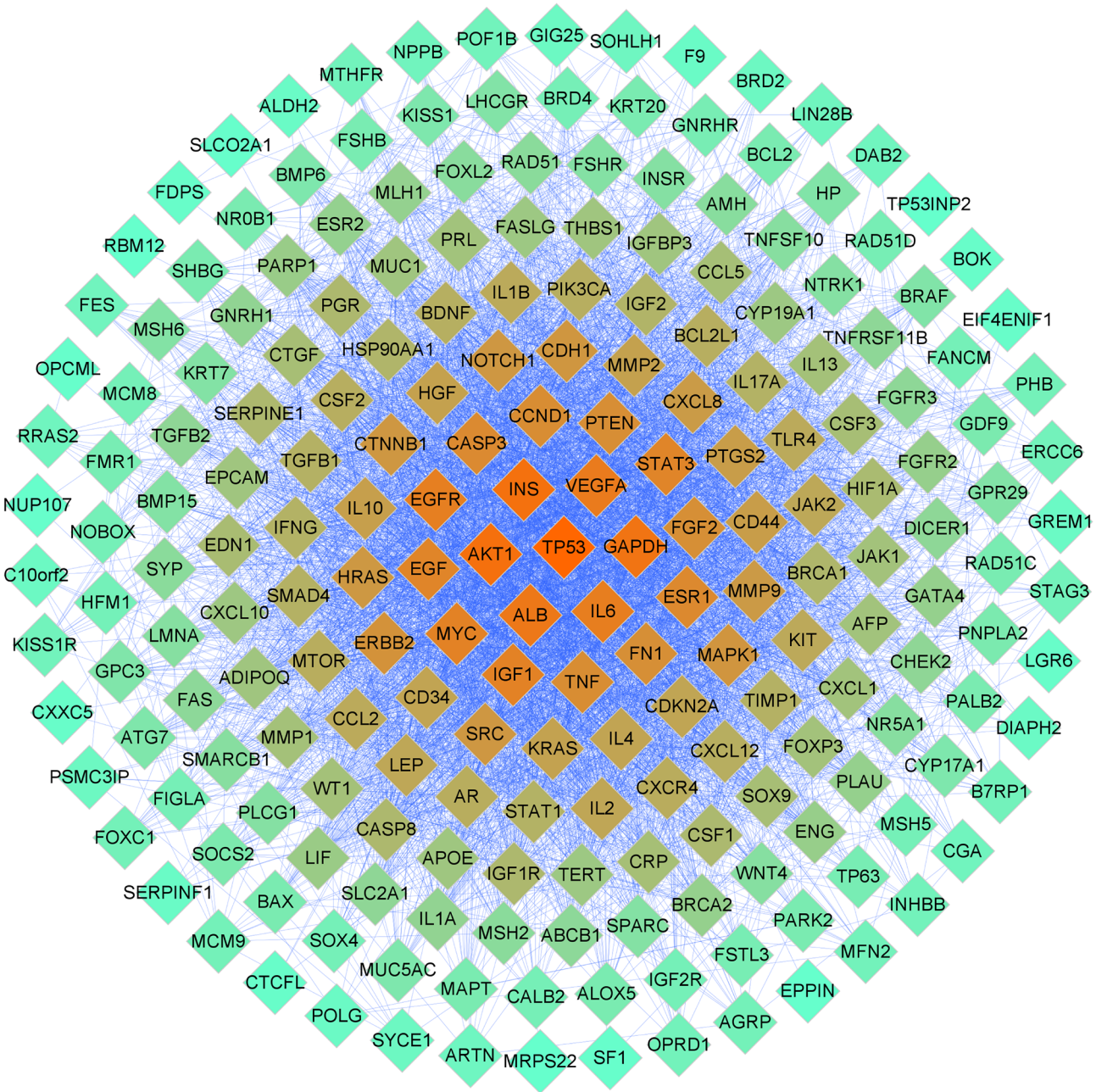

FIGURE 3 | PPI network related to DOR. The color of the nodes is illustrated from red to cyan in descending order of degree values.

comprised three pathways, including the estrogen signaling pathway (hsa04915), GnRH signaling pathway (hsa04912), and progesterone-mediated oocyte maturation (hsa04914). In addition, the pathway class for each of the 123 pathways in the KEGG database was obtained, and the number of KEGG pathways classified in different biological systems is shown in **Figure 8A**. Meanwhile, according to the pathway class, five sub-networks were constructed to explain melatonin's multi-mechanism on DOR integrally.

Molecular Docking

Eight hub genes were selected for molecular docking analysis with melatonin. The active site parameters of each target were calculated and are listed in **Table 2**. The lower docking affinity reflects the stronger binding ability between melatonin and its targets, and the binding pose with the strongest affinity was selected to analyze the interaction between melatonin and its targets. As shown in **Table 2**, except for ALB, the affinity of the remaining targets and melatonin was lower than -5 kcal/mol,

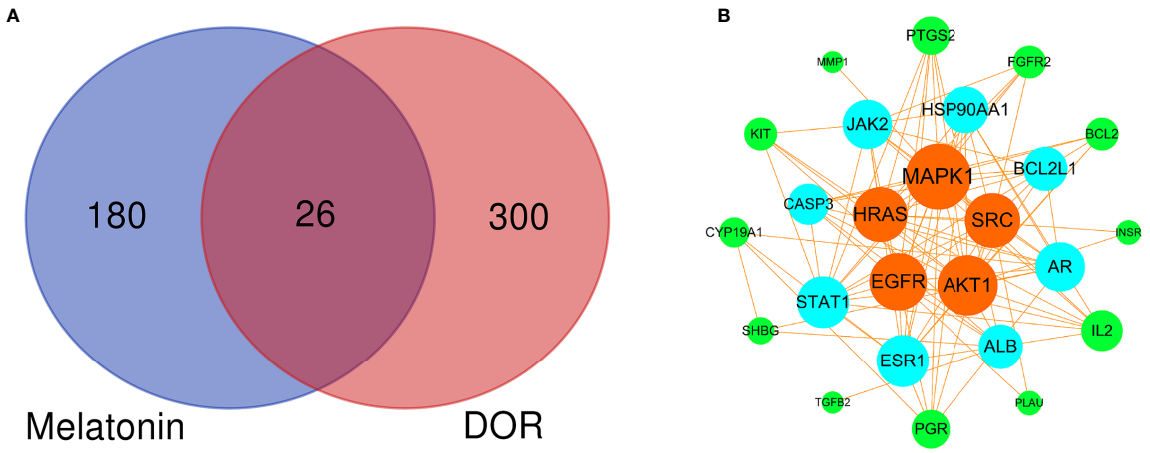

FIGURE 4 | Venn diagram and PPI network of potential therapeutic targets. **(A)** Venn diagram of intersected targets of melatonin and DOR. **(B)** PPI network of potential therapeutic targets. The node sizes and colors are illustrated from large to small and orange to green in descending order of degree values.

TABLE 1 | The KEGG results.

Pathway class	Pathway	Count	Total genes	p-value
Cell growth and death	Apoptosis	6	136	2.78E-06
Endocrine and metabolic disease	AGE-RAGE signaling pathway in diabetic complications	8	100	3.79E-10
Endocrine system	Prolactin signaling pathway	7	70	1.15E-09
Endocrine system	Thyroid hormone signaling pathway	6	121	1.40E-06
Endocrine system	Growth hormone synthesis, secretion and action	5	119	2.68E-05
Endocrine system	GnRH signaling pathway	4	93	1.73E-04
Endocrine system	Ovarian steroidogenesis	3	51	4.95E-04
Endocrine system	Estrogen signaling pathway	9	138	1.55E-10
Endocrine system	Progesterone-mediated oocyte maturation	4	100	2.29E-04
Endocrine system	GnRH secretion	3	64	9.64E-04
Immune system	IL-17 signaling pathway	5	94	8.49E-06
Signal transduction	JAK-STAT signaling pathway	8	162	1.79E-08
Signal transduction	VEGF signaling pathway	5	59	8.32E-07
Signal transduction	NF-kappa B signaling pathway	4	104	2.67E-04
Signal transduction	PI3K-Akt signaling pathway	12	354	1.30E-10
Signal transduction	MAPK signaling pathway	9	294	1.22E-07
Signal transduction	FoxO signaling pathway	6	131	2.23E-06

indicating a strong binding affinity. Therefore, melatonin may improve OR by regulating the activity of these proteins. **Figure 9** shows the binding mode of melatonin with the hub targets. Taking **Figure 9A** as an example, melatonin completely entered the active site of AKT1 and formed hydrophobic interactions with residues T291(A) and V164(A). Moreover, the formation of three hydrogen bonds between melatonin and the active site residues of AKT1 involved residues E234 (A), L156 (A), and D292 (A).

DISCUSSION

Ovarian reserve plays a crucial role in reproductive potential and endocrine stability. Driven by societal trends, many young women choose to postpone marriage and childbirth. However, their OR sharply declines after the age of 35 years (7, 50). Besides leading to reproductive dysfunction, DOR has been associated with increased risk factors for cardiovascular disease and depression (51, 52). As mentioned previously, clinical findings have confirmed that melatonin effectively improves OR, but the therapeutic mechanism of action is still not fully understood. Therefore, in the present study, for the first time, systematic and comprehensive network pharmacology was utilized to reveal the mechanism of action of melatonin against DOR and to provide relevant information for further preclinical or clinical research. According to our network pharmacology results, AKT1, EGFR, MAPK1, HRAS, SRC, ESR1, AR, and ALB play vital roles in improving OR via melatonin. Interestingly, the molecular docking of the hub genes and melatonin exhibited high affinities, implying that the eight hub genes may be highly correlated in the treatment of DOR with melatonin.

Melatonin's Eight Hub Targets in DOR

AKT1, which belongs to the AKT subfamily of serine/threonine kinases, is a multifunctional protein that regulates cell growth, survival, and proliferation (53). Emerging evidence has shown that melatonin can inhibit early follicle atresia and slow down the exhaustion of the ovarian follicle reserve by regulating the PI3K/

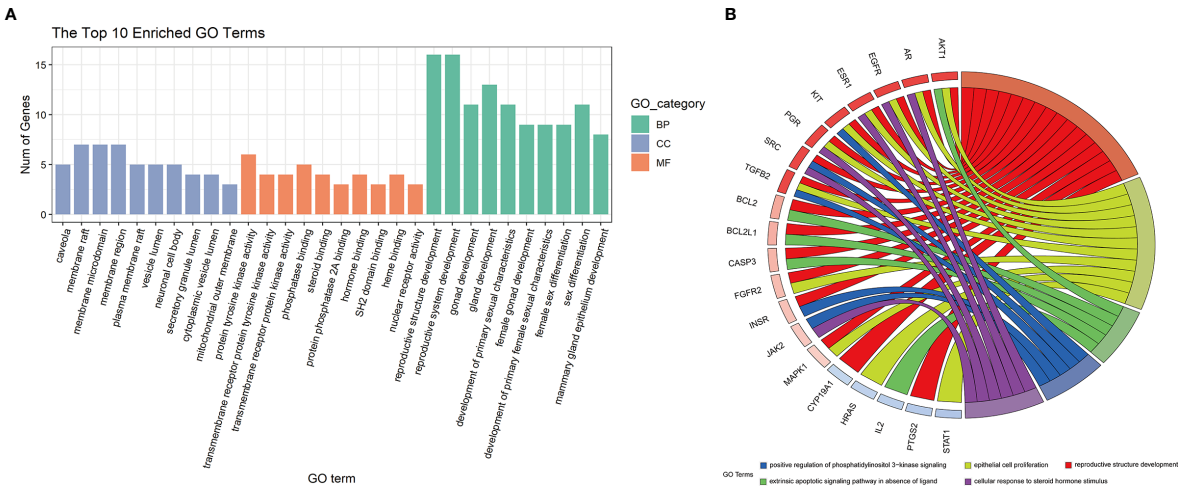

FIGURE 5 | GO enrichment analysis and the top 5 enriched biological processes. **(A)** GO enrichment analysis. The top 10 significantly enriched terms of each part. BP, biological process; CC, cell component; MF, molecular function. **(B)** The top 5 enriched biological processes.

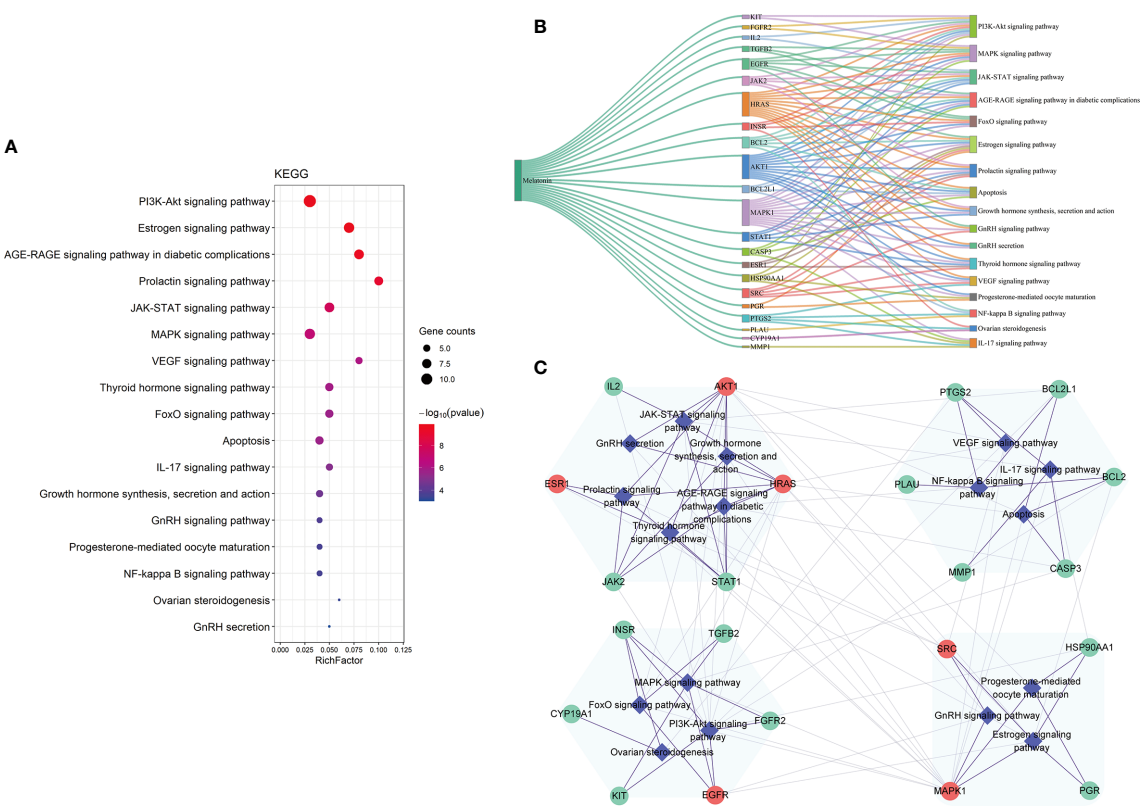

FIGURE 6 | The KEGG pathway analysis of the 26 potential therapeutic targets. **(A)** The 17 significant pathways. The bubbles' sizes are indicated from large to small in descending order of the count of the potential targets enriched in the pathways. The bubbles' colors are indicated from red to blue in descending order of -lg (*p*-value). **(B)** Melatonin-targets-pathways network. The width of the line is proportional to the number of connected points. **(C)** Module analysis of the target-pathway network. The diamond nodes represent the pathways, and the circular nodes represent the targets. The red nodes represent the hub genes obtained from the PPI network of potential therapeutic targets.

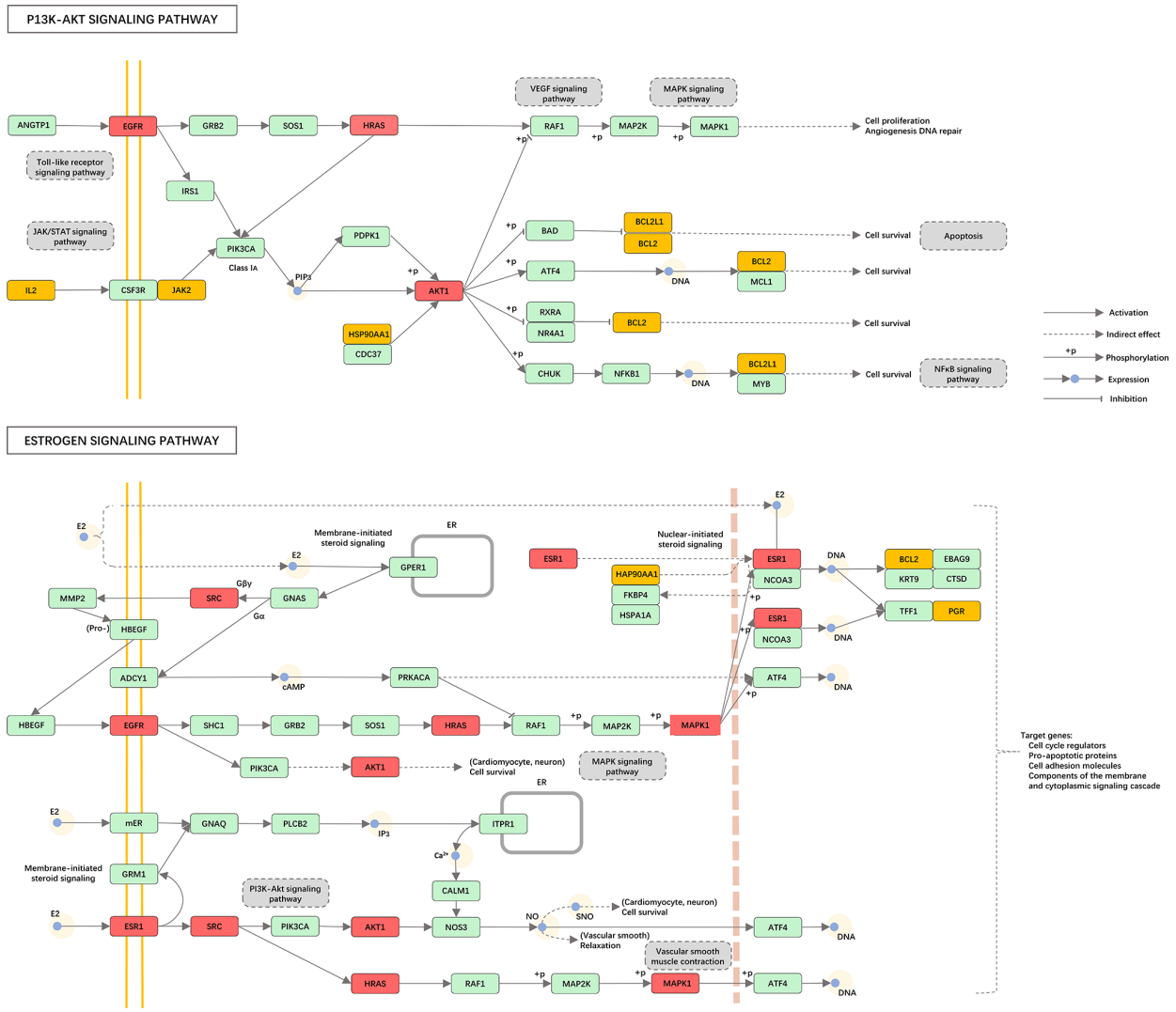

FIGURE 7 | Distribution of the potential therapeutic targets on significantly enriched pathways. The red nodes represent key genes, the yellow nodes represent overlapping targets of Melatonin and DOR targets, and the green nodes represent the other targets in estrogen signaling pathway and PI3K-AKT signaling pathway.

AKT pathway in mice (54). Similarly, Leung et al. found that melatonin acts as a modulator of ovarian function and stimulates theca cell steroidogenesis by activating the PI3K/AKT pathway in bovine small follicles (55). Additionally, melatonin can ameliorate decreased embryo development caused by the AKT1 inhibitor SH6 during the *in vitro* maturation step by enhancing oocyte maturation, cumulus cell expansion, and protection from DNA fragmentation (56).

EGFR also plays an essential role in ovarian function (57–59). LH-induced EGFR activation is an essential component for the communication between the outer mural granulosa and theca cells and the inner cumulus cells and oocytes, leading to cumulus cell expansion and oocyte maturation (60). Interestingly, Tian et al. found that melatonin can upregulate the expression levels of EGFR and effectively improve the efficiency of oocyte maturation *in vitro* (61). Tian et al. further showed that melatonin enhances the expression of EGFR in cumulus cells and improves cumulus-oocyte complex maturation, mainly via melatonin receptor 1 (62). Moreover, several studies have shown that the activation of EGFR promotes several signaling pathways, including MAPK, PI3K/AKT, and JAK/STAT pathways, all of which play a crucial role in follicle recruitment, development, and maturation (63–66). These results suggest that melatonin enhances ovarian reserve variously by upregulating EGFR levels.

MAPK1, also known as extracellular signal-regulated kinase 2 (ERK2), is a downstream effector of the EGFR pathway. Activated ERK regulates the expression of LHβ and FSHβ, which are gonadotropin synthesis genes, and induces follicle growth and ovulation (67, 68). In addition, the activation of EGFR-ERK1/2 dependent gene transcription leads to the cascade of prostaglandin E2 and p38MAPK induction, which in turn stimulates the production of EGFR ligands (AREG, EREG, and BTC) in granulosa

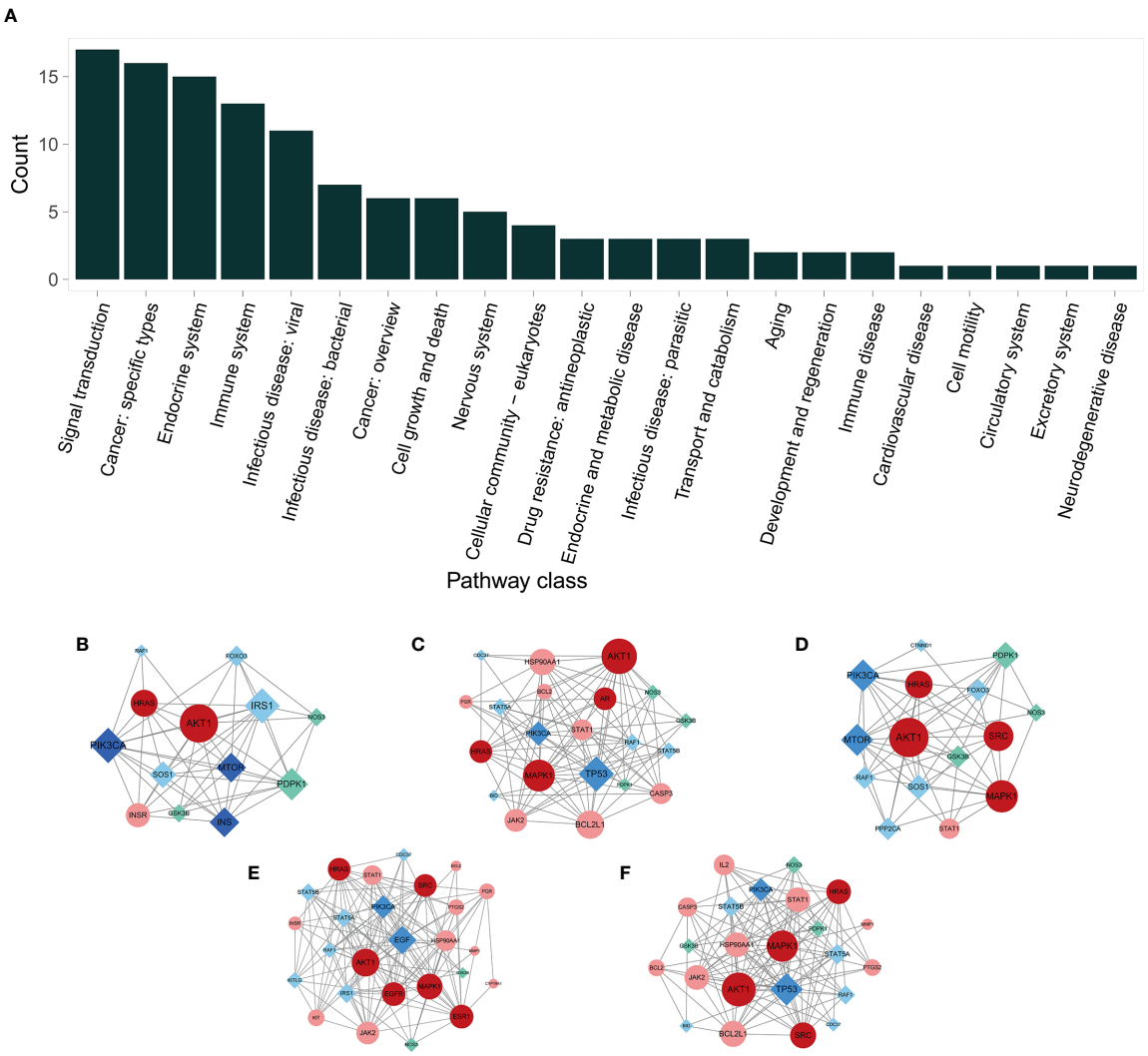

FIGURE 8 | The KEGG pathway class analysis and sub-networks in different pathway classes. **(A)** The pathway class distribution. **(B–F)** Melatonin's target sub-networks in different pathway classes. **(B)** Aging; **(C)** Cell growth and death; **(D)** Development and regeneration; **(E)** Endocrine system; and **(F)** Immune system. The circular nodes indicate the primary proteins, and the diamond nodes indicate secondary proteins. The pink nodes indicate the common targets of melatonin and DOR; the red nodes indicate the PPI network's hub genes of potential therapeutic targets; the cyan nodes indicate the melatonin targets, and the deep blue nodes indicate the DOR targets.

TABLE 2 | Docking parameters and results.

Targets	PDB ID	Box_center (x, y, z)/Å	Box_size (x×y×)/	Affinity/(kcal/mol)
AKT1	3MV5	5.1, 3.0, 17.9	16.4×15.4×14.7	-7.6
ALB	3JQZ	45.4, 8.9, -36.6	19.2×18.0×11.8	-4.9
AR	2PIU	27.5, 2.8, 5.5	16.4×19.3×13.0	-7.2
EGFR	2ITY	-48.9, -0.9, -22.9	21.4×16.1×22.5	-6.5
ESR1	1ERE	9.1, 46.2, 131.2	15.2×18.7×15.4	-7.3
HRAS	6D59	35.2, 30.2, 23.1	18.9×17.5×19.4	-7.1
MAPK1	5NHV	-15.6, 13.5, 42.4	19.9×16.0×15.6	-6.9
SRC	4K11	19.6, 23.1, 57.1	17.5×17.4×15.9	-7.2

and cumulus cells, finally activating the entire EGF network (58). For the first time (2001), Leung et al. found that melatonin via the MAPK pathway regulates progesterone production, LH receptor, GnRH, and GnRH receptor gene expression in human granulosa-luteal cells, which play a direct role in regulating ovarian function (69). Furthermore, melatonin has been shown to enhance follicle growth and proliferation in cadmium-induced injury in rat ovaries via the ERK1/2 and mTOR pathways (70).

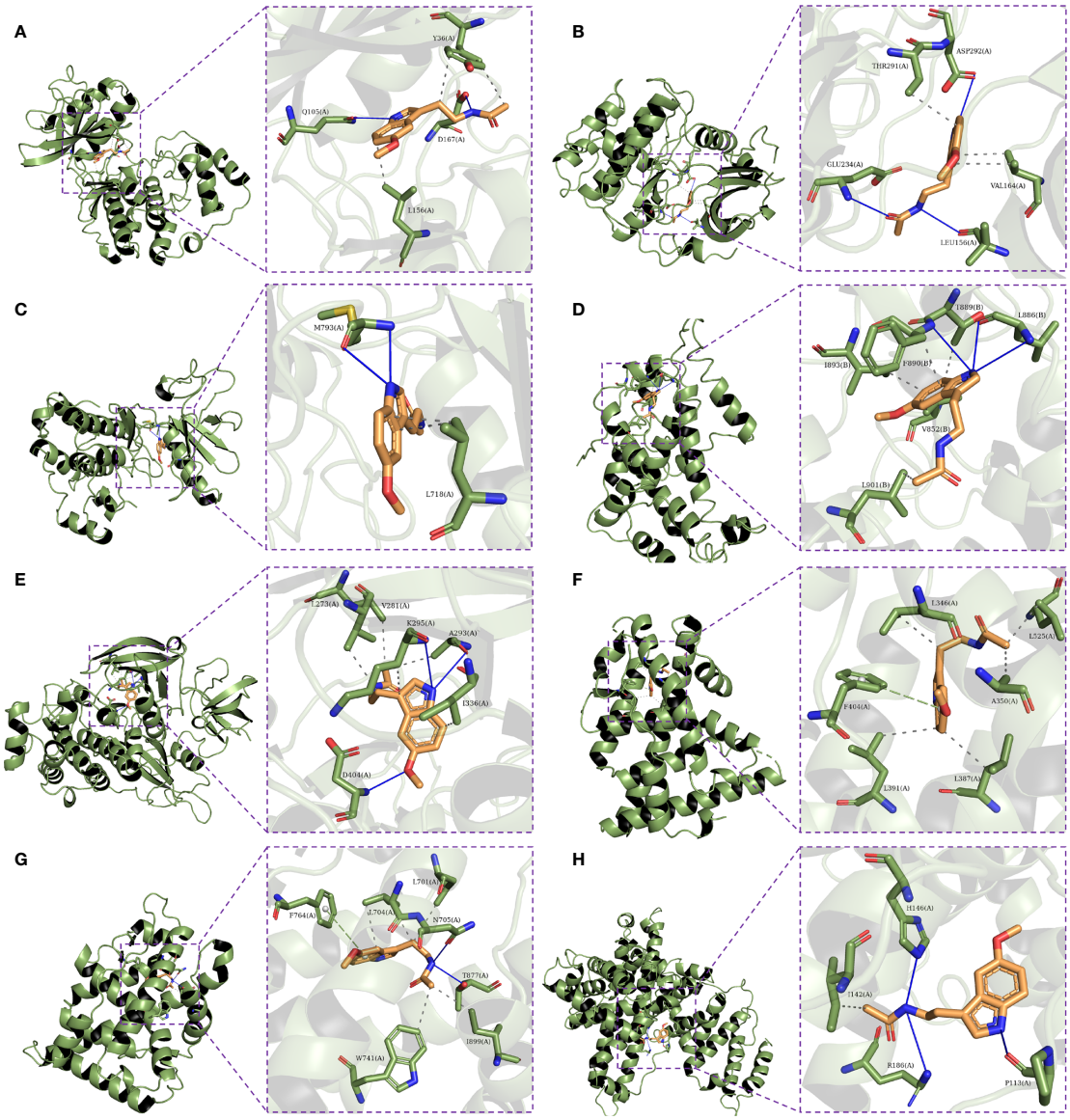

FIGURE 9 | Molecular docking of the eight hub targets with Melatonin. **(A)** The binding poses of MAPK1 complexed with melatonin. **(B)** The binding poses of AKT1 complexed with melatonin. **(C)** The binding poses of EGFR complexed with melatonin. **(D)** The binding poses of HRAS complexed with melatonin. **(E)** The binding poses of SRC complexed with melatonin. **(F)** The binding poses of ESR1 complexed with melatonin. **(G)** The binding poses of AR complexed with melatonin. **(H)** The binding poses of ALB complexed with melatonin.

As the primary female sex hormones, estrogens are responsible for maturing and maintaining the female reproductive system and are also involved in gonadotropin secretion and ovarian follicle maturation. Estrogens exert their functions by binding to ERα and ERβ, encoded by ESR1 and ESR2, respectively. SRC and HRAS, the downstream proteins of ESR1 in the estrogen signaling pathway, participate in various cellular processes, including proliferation, differentiation, and adhesion (71). Many studies support the beneficial effects of androgens in follicular development, which may be related to AR upregulating FSH receptor expression, stimulating FSH activity in GCs, and promoting follicles from the anterior sinus phase to the anal phase (72–75). In addition, although the expression pattern and role of ALB in the ovaries have not been fully clarified, as a major serum protein, ALB plays a vital role in steroid hormone (SHs) carriers and acts as a regulator of SHs' access to their receptors (76, 77). At present, no animal or clinical studies have directly confirmed that melatonin can improve OR through the above five targets (ESR1, HRAS, SRC, AR, and ALB). However, based on the molecular docking results in the current study (as well as AKT1, MAPK1, and EGFR, the above five targets have a good binding ability with melatonin) and combining their physiological roles in the ovaries, we speculate that melatonin could play a

beneficial role in ovaries via these targets. These results provide a preliminary basis and reference for future in-depth research on the mechanism of melatonin in animal models.

Important Pathways and Functional Modules of Melatonin's Putative Targets

The pathophysiological mechanism of DOR is especially complicated, and various biological processes and pathways are involved in the DOR process. The 26 therapeutic targets screened in this study mainly participate in reproductive structure development, epithelial cell proliferation, the extrinsic apoptotic signaling pathway, PI3K signaling, and response to steroid hormones. Furthermore, the KEGG pathway analysis indicated that the PI3K-Akt signaling pathway (hsa04151) and the estrogen signaling pathway (hsa04915) were the two most enriched signaling pathways (**Figure 6A**). Accumulating evidence suggests that the PI3K-Akt signaling pathway plays a key role in folliculogenesis processes, including follicle recruitment, development, and maturation (63–65). The estrogen signaling pathway is vital for the maturation and maintenance of the female reproductive system (78).

To further understand melatonin mechanisms in improving OR, the target pathway network was divided into four densely linked functional modules, as shown in **Figure 6C**. The 1st module consists of pathways in the endocrine system and related signaling pathways. The 2nd module includes pathways in cell growth and death, the immune system, and related signaling pathways, and the 3rd module is related to signal transduction. The 4th module includes pathways in the endocrine system related to the regulation of ovarian function. According to the theory of network biology, the topology of a biological network is bridged to its function (79). These modules reflected melatonin's effects on endocrine and immune regulation, anti-apoptosis, and ovarian function improvement. In addition, exogenous growth hormone administration has been shown to improve oocyte and embryo quality in IVF treatment of women with poor OR (80, 81). The functional modules analysis showed that melatonin is closely related to the synthesis, secretion, and action of GH, which also supports the function of melatonin in improving OR.

Biological Processes and Organ Systems Regulated by Melatonin's Putative Targets

Importantly, in this study, to explain the multi-mechanism of melatonin on DOR, five sub-networks were constructed. The aging sub-network (**Figure 8B**) showed that melatonin targets AKT1, mTOR, and PIK3A, among others. The AKT/TOR pathway is a recognized central signaling pathway regulating lifespan, highlighting the anti-aging effect of melatonin (82, 83). Consistently, previous studies have shown that melatonin can prolong the lifespan and delay ovarian aging in mice (84, 85).

Apoptosis is a critical biological process that plays a vital role in germ cell depletion in mammalian ovaries (86). Follicular atresia caused by GC apoptosis is the primary process responsible for follicle loss (87–89). Bcl2-like-proteins are anti-apoptotic factors that may inhibit apoptosis. In the subnetwork of cell growth and death (**Figure 8C**), melatonin acts on BCL2, BCL2L1, BID, and CASP3, suggesting that melatonin exerts anti-apoptotic effects.

The sub-network of development and regeneration (**Figure 8D**) suggests the effect of melatonin in follicle development regulation. This network includes AKT, MAPK1, and HARS, which are involved in follicle growth and survival (90).

In addition to maintaining homeostasis, the immune system is associated with modulation at every level of the hypothalamic-pituitary-ovarian axis, as well as the regulation of proliferation and differentiation of ovarian germline stem cells (91). AKT1 and its interactions with MAPK1, JAK2, STAT1, etc., are involved in regulating melatonin in both the endocrine and immune systems (**Figures 8E, F**). Although they are not immune genes, they play an essential role in the division, differentiation, development, and function of various types of immune genes and immunomodulatory cytokines, including T-cells, IFN, Th17, and dendritic cells (92–94).

CONCLUSIONS

In summary, melatonin may improve OR by intervening in a series of targets (such as AKT1, EGFR, MAPK1, HRAS, SRC, ESR1, AR, and ALB), biological processes (reproductive structure development, epithelial cell proliferation, extrinsic apoptotic signaling pathway, PI3K signaling, and response to steroid hormone), and signaling pathways (such as PI3K-Akt and estrogen signaling pathways). Melatonin could exhibit anti-aging, anti-apoptosis, endocrine, and immune system regulation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

QZ conceptualized the manuscript. LY, HX, YC, YX, CM, and YZ collected the literature, wrote the manuscript, and made the figures. QZ edited and made significant revisions to the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.630504/full#supplementary-material

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MINI REVIEW
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The Role of Androgen Supplementation in Women With Diminished Ovarian Reserve: Time to Randomize, Not Meta-Analyze

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The management of patients with diminished ovarian reserve (DOR) remains one of the most challenging tasks in IVF clinical practice. Despite the promising results obtained from animal studies regarding the importance of androgens on folliculogenesis, the evidence obtained from clinical studies remains inconclusive. This is mainly due to the lack of an evidence-based methodology applied in the available trials and to the heterogeneity in the inclusion criteria and IVF treatment protocols. In this review, we analyze the available evidence obtained from animal studies and highlight the pitfalls from the clinical studies that prevent us from closing the chapter of this line of research.

Keywords: androgens, testosterone, DHEA, poor ovarian response (POR), diminished ovarian response (DOR)

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INTRODUCTION

In women, testosterone and dihydrotestosterone (DHT), the bioactive androgens that bind directly to the androgen receptor (AR), are produced by peripheral conversion of androgen precursors (androstenedione, dehydroepiandrosterone and dehydroepiandrosterone sulfate) that are secreted from both the ovary and adrenal gland (1, 2).

The AR is expressed at all levels of the female hypothalamic-pituitary-gonadal axis (2). In the ovary, the AR has been detected in several stages of oocyte development from the primary stage onwards, as well as in the ovarian stroma (3). The fact that hyperandrogenic women present an increased number of small antral follicles suggests a role for androgens in both follicular development and follicular arrest. Clinical examples of this effect include polycystic ovarian syndrome (PCOS) and congenital adrenal hyperplasia patients (4). On the other hand, although initial studies using histomorphologic criteria suggested that exposure to exogenous testosterone treatment in female-to-male transexual patients induced polycystic ovary morphology (5, 6), more recent studies using both histologic and ultrasound criteria have not confirmed these findings (7–9).

Circulating androgen levels have been reported to decline with age, especially during the earlier reproductive years (10). Similarly, the reproductive aging process consists of a gradual reduction in oocyte quantity and quality, with a consequent age-related decrease in the reproductive potential (11, 12). In the light of these findings, IVF centers have initiated androgen pretreatment in patients with diminished ovarian reserve, intending to improve their reproductive outcomes. In fact, a recent

survey has shown that more than 40% of physicians in Europe and Australia are prescribing off-label androgens in this subgroup of patients (13). However, the evidence for including this approach in our clinical practice is scarce.

The aim of this review is to analyze the available evidence from animal studies regarding the impact of androgen supplementation on folliculogenesis, as well as the drawbacks from clinical studies that might preclude the obtention of definitive conclusions to guide an evidence-based approach for such a challenging population.

METHODS

The Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE *via* PubMed, the Web of Science and Scopus were screened with a combination of keywords related to ART, poor responders, diminished ovarian response, androgens, testosterone and DHEA in various combinations. The search period was from the date of inception of each database until 1 December 2020. Only full text papers published in English were included.

THE PROMISING EVIDENCE FROM ANIMAL STUDIES

Primordial Follicle Initiation

Previous studies in primates have shown that androgens increase the numbers of small- and medium-sized follicles but not large preovulatory follicles (14). In particular, testosterone and DHT pretreatment increased the number of primary follicles. Also, they resulted in a significant increase in insulin growth factor I (IGF-I) and IGF-I receptor mRNAs in the oocytes of primordial follicles, suggesting that androgen-induced activation of oocyte IGF-I signaling may trigger primordial follicle growth (15). More recently, mouse studies have corroborated that testosterone promotes primordial follicle to primary follicle transition *via* an AR-mediated pathway rather than by transformation into estradiol (16).

Preantral to Antral Stage Transition

Besides the effect on primordial follicle initiation, androgens also seem to have a role in the preantral to antral stage transition. In vivo studies in ovine models have shown that DHEA exposure stimulates early follicular growth during the preantral and early antral follicular stages (17). Studies in mouse models have also shown that both DHT and testosterone stimulate granulosa cell (GC) proliferation and both secondary and preantral follicle growth (18). Moreover, androgens seem to support follicle development during the FSH-dependent preantral stage by increasing the expression of FSH receptor mRNA levels and, therefore, enhancing FSH action (19, 20). GC-specific AR-null mice experiments have also shown that AR signaling in GCs is necessary for progression beyond the preantral stage (21). Androgens enhance antiapoptotic pathways, thereby contributing to follicle survival, and improve sensitivity to FSH-induced follicle growth and progression to the antral stage (22). On the other hand, when AR signaling is blocked, preantral follicles cannot progress to antral follicles and, instead, are subjected to an increased rate of atresia.

The Peri-Ovulatory Stage

The effect of androgens in later stages of follicle development, namely in the pre- and peri-ovulatory stage, is controversial. Studies in primates have shown that testosterone treatment did not increase the number of preovulatory follicles (14). However, experiments in pigs have shown that androgens might have regulatory functions during late follicular development (23). In fact, DHT treatment resulted in an increase in the amount of FSH receptor mRNA in preovulatory follicles and increased ovulation rate (23). Similarly, experiments in mice have also shown that testosterone has a role in the maturation of oocytes arrested in prophase I of meiosis (24) and that DHT significantly increased the number of ovulated oocytes (22). On the other hand, Romero and Smitz reported that elevated levels of androstenedione and testosterone negatively affected meiotic resumption (25). These conflicting findings regarding the role of androgens in the late stages of follicular development suggest that further studies are needed to clarify the physiopathology behind such complex interactions.

Figure 1 highlights the main androgen effects on folliculogenesis.

Genetic Studies

Finally, data from genetic models have also reaffirmed the role of AR-mediated activity in the regulation of ovarian function. Studies using female mouse models homozygous for an inactivated AR (ARKO) have revealed reduced fertility and a defective folliculogenesis (26–28), as well as a reduced litter size (27), increased follicular atresia and premature ovarian failure (21). Together, these data suggest the AR signaling pathway mediates both intra and extra-ovarian actions, with an essential role in maintaining normal ovarian function and fertility.

THE PITFALLS FROM CLINICAL STUDIES

All these promising data obtained from animal studies and the fact that both androgens and ovarian reserve decline steeply with age, led to the speculation that androgen replacement in women with DOR might delay these age-related effects. However, despite several lines of evidence supporting a role for androgens in folliculogenesis, the available data from clinical studies remains unconvincing. This might be related to the methodological inconsistencies observed in the available trials (**Tables 1** and **2**).

Dehydroepiandrosterone

A case series of five patients with unexplained infertility and previous poor response to ovarian stimulation was the first study to analyze the effect of DHEA pretreatment on ovarian response (51). In this study, 80 mg/day of oral micronized DHEA was given for 2 months, after which ovarian stimulation was started

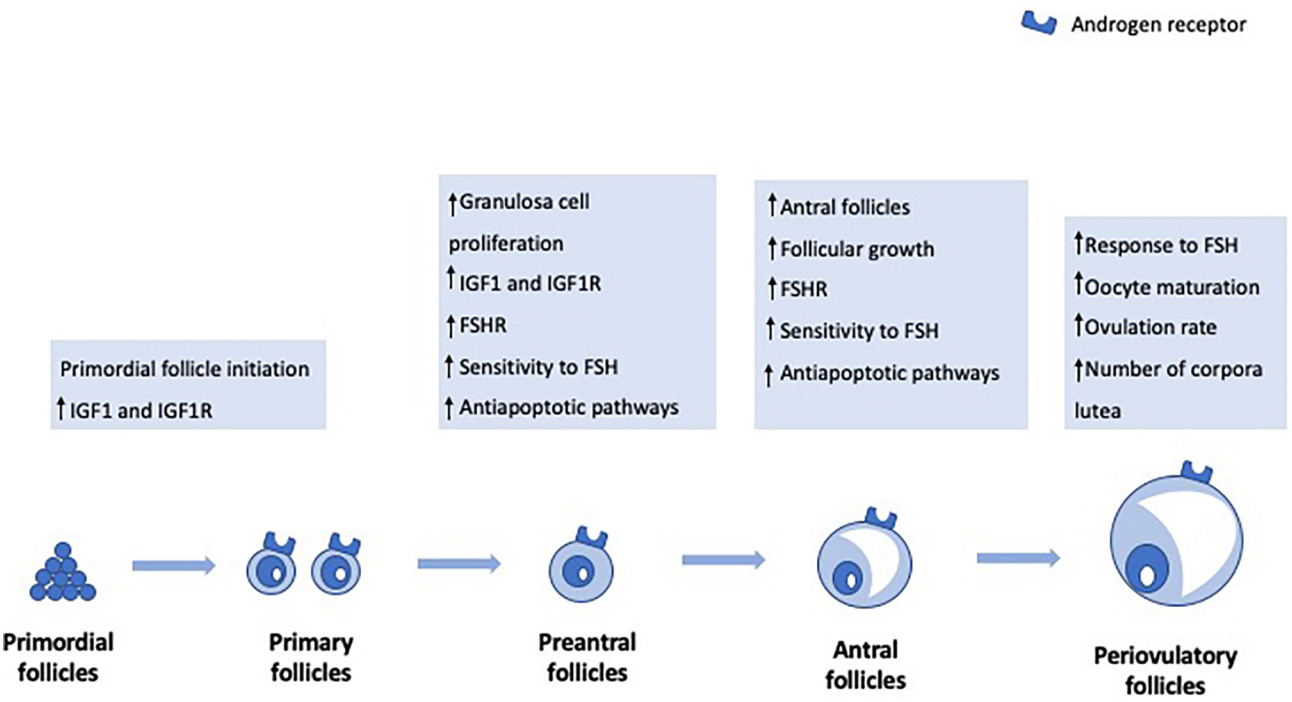

FIGURE 1 | Androgen effects on folliculogenesis.

with recombinant follicle stimulating hormone (rFSH) for intrauterine insemination. The authors concluded that oral DHEA supplementation might improve ovarian response and reduce gonadotrophin consumption. Five years later, a case report of a 43-years old patient seeking embryo accumulation for preimplantation genetic screening draw the scientific community's attention to the role of androgens in ovarian response to stimulation (52). After her first stimulation cycle, the patient started self-administering 75 mg/day of oral micronized DHEA and initiated acupuncture treatment. In total, the patient performed 9 stimulation cycles with different stimulation protocols, and a significant increase in ovarian response was reported after four months of DHEA pretreatment. Since then, multiple observational and randomized controlled trials have followed, with varying DOR and poor ovarian reserve (POR) definitions, with DHEA doses ranging from 50 to 90 mg/day and a treatment duration ranging from 1 to 12 months, both before and during controlled ovarian stimulation (**Tables 1** and **2**). Importantly, no pharmacological studies have been performed to determine the optimal dose, duration or timing of DHEA supplementation in DOR patients.

Another key limitation regarding many studies on DHEA pre-treatment is the frequent use of patients as their own controls, comparing ovarian response after DHEA supplementation with a previous cycle. This study design does not take into account the importance of biological variability in the response to ovarian stimulation and the natural process of the regression to the mean, precluding definitive conclusions regarding the true effect of such treatment (77).

Also noteworthy is the fact that oral DHEA formulations are dietary supplements and therefore are not regulated by the US Food and Drug Administration (FDA) nor by the European Medicines Agency (EMA) and are exempt from pharmaceutical quality standards. Consequently, the true standardization of the formulations used cannot be guaranteed (78).

Testosterone

Numerous observational and randomized controlled trials have also been published on the use of testosterone pre-treatment on POR and DOR patients (**Tables 1** and **2**). Most studies report the use of transdermal testosterone, both in gel and patches, with doses of treatment based on Vendola's studies on primates (14, 15). In these studies, an effect on follicular development was reported with transdermal testosterone 20 μg/Kg/day, obtained with a 12.5mg/day gel application or a 2.5mg/day patch. Importantly, however, pharmacokinetics studies performed in postmenopausal women revealed that the administration of 4.4-5 mg testosterone gel or cream raised free testosterone levels within the reference range for reproductive-aged women whereas higher doses increased testosterone levels above the physiological range (79, 80). These findings question the potential clinical benefit (or harm) of using the high doses that have been reported so far.

The issue of the duration of treatment has also been another point of conflict in the published studies, ranging from 5 days, based on Vendola's studies (14, 15), to 21-28 days, based on a RCT that reported that testosterone effects at the follicular level occurred after at least three weeks of testosterone pre-treatment (32).

TABLE 1 | Published randomized controlled trials on the use of DHEA and Testosterone in DOR and POR patients.

Author *Year*	Definition of POR	Number of patients	Dose	Duration	Stimulation protocol	Primary outcome
Testosterone						
Massin et al. (29) *2006* *	Previous POR (Peak E2<1200pg/mL and ≤5 oocytes) and D3 FSH > 12 IU/L or E2 > 70pg/mL or Inhibin B <45ng/mL	49	10 mg/d	15-20 d	NR	Total number of retrieved oocytes
Fábregues et al. (30) *2009*	Previous POR and 31-39y	62	20 ug/kg/d	5 d	Long GnRH agonist	Incidence of low responders
Kim et al. (31) *2011*	Previous cycle with ≤3 oocytes retrieved despite high Gn dose	110	12.5 mg/d	21 d	GnRH antagonist	Number of MII oocytes retrieved
Kim et al. (32) *2014*	Previous cycle with ≤3 oocytes retrieved despite high Gn dose	120	12.5 mg/d	I1: 14 d/ I2: 21 d/ I3: 28 d	GnRH antagonist	Number of MII oocytes retrieved
Marzal Escrivá et al. (33) *2015*	≥2: ≥38y, AFC ≤6, FSH ≥10 IU/L, AMH ≤5pg/mL AND ≤4 follicles of ≥16 mm on the day of trigger or E2 ≤500 pg/mL on the day of trigger or ≤ 4 MII	66	20 ug/kg/d	7 d	GnRH antagonist	Number of MII oocytes retrieved
Bosdou et al. (34) *2016*	Bologna criteria	50	10 mg/d	21 d	Long GnRH agonist	Total number of retrieved oocytes
Saharkhiz et al. (35) *2018* *	Bologna criteria	48	25 mg/d	During COS	GnRH antagonist	NR
DHEA						
Wiser et al. (36) *2010*	<5 oocytes retrieved in previous cycle; poor quality embryos; previous cycle cancelation due to poor response with rFSH 300IU	33	75 mg/d	> 6 weeks	Long GnRH agonist	Peak estradiol levels, the number of retrieved oocytes, embryo quality and number of embryos reserved for transfer
Artini et al. (37) *2012*	Bologna criteria	24	75 mg/d	12 weeks	GnRH antagonist	HIF1 and VEGF concentrations in the FF and the number of MII oocytes
Moawad and Shaeer (38) *2012*	<40y; <5 oocytes retrieved in previous cycle; previous cycle cancelation due to poor response with rFSH 300IU; AMH<1.7ng/mL	133	75 mg/d	>12 weeks	GnRH antagonist	Peak E2 levels, number of retrieved oocytes and number of embryos
Yeung et al. (39) *2013* *	POI	22	75 mg/d	16 weeks	NA	Serum AMH level
Yeung et al. (40) *2014* *	<40y, subfertility >1y and AFC<5	32	75 mg/d	12 weeks	GnRH antagonist	The primary outcome was the AFC at 12 weeks
Kara et al. (41) *2014*	AMH<1ng/mL or FSH>15IU/L and AFC < 4	208	75 mg/d	12 weeks	Microdose flare	NR
Zhang et al. (42) *2014*	D3 FSH ≥ 10IU/L or FSH/LH>3; AFC<5; previous cycle with <5 oocytes retrieved or previous cancelled cycle due to POR	95	75 mg/d	12 weeks	HMG + Clomiphene citrate	Follicular fluid BMP- 15 and GDF-9 and serum AMH, FSH and E2
Kotb et al. (43) *2016*	Bologna criteria 25-40y	140	75 mg/d	3 months	GnRH antagonist	Clinical pregnancy rate
Agarwal et al. (44) *2017* *	18-45y with DOR: (1) FSH levels >7 mIU/ml for age<33y; >7.9 mIU/ml for age 33–37y; >8.4 mIU/ml for age >38 years. (2) AMH < 1.05 ng/ml. (3) AFC<4	40	75 mg/d	12 weeks	NA	AMH, FSH and AFC
Narkwichean et al. (45) *2017* *	AFC<10 and/or AMH <5 pmol/L	52	75 mg/d	>12 weeks	Long GnRH agonist	Number of oocytes retrieved
Elprince et al. (46) *2020* *	(1) serum AMH < 1.1 ng/mL, (2) FSH ≥ 10 mIU/L and ≤ 15 mIU/L on cycle D3, and (3) AFC ≤ 4	50	75 mg/d	2 Continuous cycles	Ovulation induction	NR

** Placebo controlled.*

AFC, antral follicle count; AMH, antimullerian hormone; BMP-15, bone morphogenetic protein-15; d, day(s); E2, estradiol; FF, follicular fluid; FSH, follicle stimulating hormone; GDF-9, growth differentiation factor-9; Gn, gonadotropin; GnRH, gonadotropin releasing hormone; HIF, Hypoxia inducible factor; MII, mature oocytes; NR, not reported; NA, not applicable; POI, premature ovarian insufficiency; POR, poor ovarian responders; VEGF, vascular endothelial growth factor; y, years.

TABLE 2 | Published observational trials on the use of DHEA and Testosterone in DOR and POR patients.

Author *Year*	Study design	Definition of POR	Number of patients	Dose	Duration	Stimulation protocol	Main outcome measure
Testosterone							
Balasch et al. (47) *2006*	Prospective self-controlled	31-39y patients undergoing their third IVF attempt with 1 or 2 previous IVF cycles cancelled because of poor follicular response, with basal FSH <10IU/L	25	2.5mg/d Patch	5 d	Long GnRH agonist	NR
Mitri et al. (48) *2016*	Retrospective	At least one previous failed or cancelled IVF cycle with suspected Gn resistance (serum FSH ≥20 mIU/L on D7) and absent or minimal follicular growth during the current cycle.	26	25mg/d gel	variable	Microflare GnRH agonist with interrupted FSH	NR
Doan et al. (49) *2017*	Prospective	History or probability of POR: AFC<5–7 or AMH≤ 1.26 ng/ml)	110	12.5mg/d gel	28 d	GnRH antagonist	NR
Fabregues et al. (50) *2019*	Retrospective	Bologna criteria	141	2.5mg/d Patch	5 d	GnRH antagonist and Long GnRH agonist	NR
DHEA							
Casson et al. (51) *2000*	Case series	Previous POR to vigorous Gn stimulation (peak estradiol ≤500 pg/ml, MII ≤2)	5	80mg/d	2 months	Ovulation induction	NR
Barad and Gleicher (52) *2005*	Case report	43y patient	1	75 mg/d	11 months	GnRH agonist	Peak E2 concentration, oocytes retrieved, and cyropreservable embryos.
Barad and Gleicher (53) *2006*	Retrospective self-controlled	Prior IVF cycle with age-appropriate COS, and < 4 oocytes retrieved, uniformly poor embryo quality and FSH >10 mIU/ml or E2 >75 pg/ml	25	75 mg/d	17.6 ± 2.13 weeks	GnRH agonist	NR
Barad et al. (54) *2007*	Retrospective	Basal FSH <12 mIU/ml, but exceeding the 95% CI of the mean value for the patient's age group or vasal FSH ≥12 mIU/ml and/ or a baseline estradiol level ≥75 pg/ml	190	75 mg/d	3.8 ± 0.3 months	Microflare GnRH agonist	Clinical pregnancy rate
Mamas and Mamas (55) *2009*	Case series	POI	5	50-75 mg/d	2-6 months	NA	NR
Mamas and Mamas (56) *2009*	Case series	POI	14	50-75 mg/d	3-7 months	NA	NR
Sonmezer et al. (57) *2009*	Prospective self-controlled	(i) cycle cancellation due to E2<130 pg/ml on cycle D6 or <450 pg/ml on the day of trigger, (ii) <4 retrieved oocytes despite vigorous ovarian stimulation.	19	75 mg/d	90-180 d	GnRH antagonist	Antral follicle count, number of follicles >14 and >17 mm on the day of HCG administration, E2 on the day of HCG administration, number of retrieved oocytes, mean number of MII, number of transferred embryos and rates of fertilization, implantation, pregnancy, and clinical pregnancy.
Gleicher et al. (58) *2009*	Retrospective	Definition of POR changed over the study period	73	75 mg/d	> 2 months	NR	Miscarriage rate
Gleicher et al. (59) *2010*	Retrospective	Elevated age-specific baseline FSH or abnormally low age-specific AMH	66	75 mg/d	>4 weeks	Microflare GnRH agonist	Number and percentage of aneuploid embryos
Gleicher et al. (60) *2010*	Retrospective	Elevated age-specific baseline FSH or universal AMH < 0.8 ng/ml	120	75 mg/d	73 ± 27 d	NA	AMH

(Continued)

TABLE 2 | Continued

Author *Year*	Study design	Definition of POR	Number of patients	Dose	Duration	Stimulation protocol	Main outcome measure
Weissman et al. (61) *2011*	Retrospective self-controlled	>1 of the following characteristics in a previous cycle with high-dose Gn stimulation:< 5 oocytes retrieved, ≤ 3 follicles ≥ 16 mm on the day of cycle cancelation, or E2 level <500 pg/ml on the day of trigger	15	75 mg/d	~3 months	NR	Progesterone concentration on day 5 of stimulation and on the day of hCG administration.
Fusi et al. (62) *2013*	Prospective	Cohort 1: Previous IVF cycle with POR Cohort 2: > 40y and DOR (AFC < 4, FSH > 10 IU/ml, AMH < 1 ng/ml	101	75 mg/d	> 3 months	Long GnRH agonist	Spontaneous pregnancies
Hyman et al. (63) *2013*	Prospective self-controlled	At least one previous IVF cycle with ≤ 4 oocytes retrieved despite high dose Gn (≥ 450IU/day)	43	75 mg/d	>3 months	NR	NR
Singh et al. (64) *2013*	Prospective self-controlled	Poor ovarian response in the previous IVF cycle(s)	31	75 mg/d	4 months	NR	AMH, FSH and antral follicle count
Yilmaz et al. (65) *2013*	Prospective	AFC <5 or AMH <1.1 ng/ml and a previous poor ovarian response	41	75 mg/d	> 6 weeks	GnRH antagonist	AMH, Inhibin B and antral follicle count
Jirge et al. (66) *2014*	Prospective self-controlled	Bologna criteria <40ys with 1 previously failed IVF cycle	31	75 mg/d	> 2 months	GnRH antagonist	Dose and duration of gonadotropin therapy, oocyte yield, embryo number and quality, pregnancy and live birth rate.
Xu et al. (67) *2014*	Retrospective	Bologna criteria	386	75 mg/d	90 d	GnRH antagonist	Ongoing pregnancy rate and implantation rate
Zangmo et al. (68) *2014*	Prospective self-controlled	<42 years, with <5 oocytes retrieved in previous IVF cycles, D2 FSH 10–20 mIU/ml	50	75 mg/d	4 months	NR	Oocyte and embryo number and quality
Tsui et al. (69) *2015*	Prospective self-controlled	Bologna criteria	10	90 mg/d	12.2 weeks	GnRH antagonist	Total doses of rFSH, days of stimulation, oocytes retrieved, fertilized oocytes, Day 3 embryos, and transferred embryos
Vlahos et al. (70) *2015*	Prospective	At least 2 of the following: >40 years, D2 FSH >9.5 mIU/ml, AMH< 2 ng/ml, at least one previous cycle of COS with < 3 oocytes retrieved, at least one cancelled attempt owing to POR and E2 < 500 pg/ml on the day of trigger	161	75 mg/d	> 3 months	GnRH antagonist	Live birth rate
Hu et al. (71) *2017*	Prospective	<40 years, subfertility >1 year, and DOR (two or more items such as FSH 10-25 IU/L, E2 >80 pg/ml, AMH <0.5-1.1 ng/ml and AFC ≤5 on cycle D2-3	106	75 mg/d	8 weeks	GnRH antagonist	NR
Chern et al. (72) *2018*	Retrospective	Bologna criteria or 2 episodes of a previous POR after maximal stimulation alone	151	90 mg/d	3 months	GnRH antagonist	Number of oocytes retrieved and clinical pregnancy rate
Al-Turki et al. (73) *2018*	Prospective	Bologna criteria, 25-40y with previously failed IVF cycle	62	50 mg/d	3 months	GnRH antagonist	Number of oocytes retrieved, fertilization rate, number of embryos and pregnancy rate
Wong et al. (74) *2018*	Prospective	POI	31	75 mg/d	12 months	NA	AMH
Chen et al. (75) *2019*	Retrospective	POSEIDON group 4	297	90 mg/d	3 months	GnRH antagonist	Number of oocytes retrieved and MII
Ozcil (76) *2020*	Retrospective	6 POI and 28 POR according to the Bologna criteria	34	50 mg/d	5 months	NA	Spontaneous clinical pregnancy rate

AFC, antral follicle count; AMH, antimullerian hormone; CI, confidence interval; COS, controlled ovarian stimulation; d, day(s); E2, estradiol; FSH, follicle stimulating hormone; Gn, gonadotropin; GnRH, gonadotropin releasing hormone; HCG, human chorionic gonadotropin; IVF, in vitro fertilization; MII, mature oocytes; NR, not reported; NA, not applicable; POI, premature ovarian insufficiency; POR, poor ovarian responders; y, years.

This should come as no surprise, if we consider that the progression from a primordial follicle to a periovulatory follicle takes approximately 3 months (81).

Too Much Is Not Enough

The vast bulk of published original studies and meta-analysis on the use of androgens pre-treatment in DOR and POR patients is depicted in **Figure 2**. One of the limitations in analyzing the effect of these adjuvant strategies in DOR/POR patients is the definition of diminished and poor response itself. In this context, the Poseidon Group introduced the concept of 'low prognosis patients' and highlighted the need for tailored evidence-based clinical algorithms for each of the four proposed risk groups (82, 83). Standardizing the inclusion criteria of future studies based on these risk groups might be a further step in minimizing study heterogeneity.

Despite the above-mentioned methodological limitations and the heterogeneity among the inclusion criteria and treatment protocols, original studies continue to be published in an attempt to optimize the clinical management of such a challenging population. With the same goal, a disproportionate number of meta-analysis has been published, especially when considering the number of original studies. **Table 3** describes the meta-analysis published on the use of DHEA and testosterone supplementation in IVF and the study design of the included trials. If we consider the low level of evidence of some of the included study designs, the lack of evidence-based protocols for both DHEA and testosterone supplementation, the heterogeneity in the definition of POR and DOR and the diversity in the IVF protocols used in the different trials, the clinical impact of the conclusions drawn from these meta-analysis might be called into question. In this regard, an individual patient data approach could be of use in increasing the strength of the available evidence.

However, to break this vicious cycle, we are left with the need to write the story of androgens supplementation in patients with DOR/POR from the beginning. In order to do so, evidence from pharmacokinetics studies (79) as well as from the timespan of human folliculogenesis (97) must be taken into account in what concerns the optimal dose and duration of treatment. In this respect, the currently ongoing multicenter double-blind placebo-controlled randomized controlled trial T-TRANSPORT (NCT02418572, available at http://clinicaltrials.gov/ct2/show/NCT02418572) might shed some light on this subject. With an intervention group undergoing 5.5 mg daily transdermal testosterone for two months prior to an IVF cycle and powered

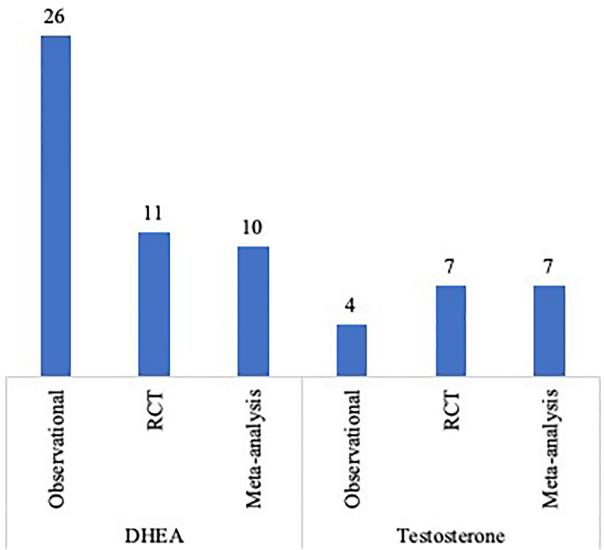

FIGURE 2 | Published original studies and meta-analysis on the use of DHEA or testosterone supplementation in POR and DOR patients.

TABLE 3 | Published meta-analysis on the use of DHEA and Testosterone in IVF.

Author	Year	Number of studies	Population	Study design
DHEA				
Narckwichean et al. (84)	2013	3	DOR/POR	1 RCT, 2 Retrospective
Li et al. (85)	2015	8	DOR/POR	2 RCT, 2 Prospective, 4 Retrospective
Qin et al. (86)	2016	9	DOR/POR	4 RCT, 2 Prospective, 3 Retrospective
Liu et al. (87)	2017	6	NOR/DOR/POR	6 RCT
Schwarze et al. (88)	2018	5	DOR/POR	2 RCT, 1 Prospective, 2 Retrospective
Xu et al. (89)	2019	9	NOR/DOR/POR	9 RCT
Testosterone				
González-Comadran et al. (90)	2012	3	DOR/POR	3 RCT
Luo et al. (91)	2014	3	DOR/POR	3 RCT
Noventa et al. (92)	2019	7	DOR/POR	7 RCT
Testosterone and DHEA				
Sunkara et al. (93)	2011	5	DOR/POR	4 RCT, 1 Retrospective
Bosdou et al. (94)	2012	3	DOR/POR	3 RCT
Nagels et al. (95)	2015	17	NOR/DOR/POR/POI	17 RCT
Zhang et al. (96)	2019	4	POR	4 RCT

DHEA, dehydroepiandrosterone; DOR, diminished ovarian reserve; NOR, normoresponders; POI, premature ovarian insufficiency; POR, poor ovarian responders; RCT, randomized controlled trials.

with clinical pregnancy rate as the primary outcome measure, this trial is expected to clarify the role of androgens in IVF.

CONCLUSION

Despite the vast amount of available literature on the use of DHEA and testosterone in POR patients, the bulk of evidence is still limited to draw definite conclusions. More than reviewing the available data and publishing new studies based on the same pitfalls, we urge to restart this chapter with well-designed clinical trials.

AUTHOR CONTRIBUTIONS

AN designed the study, performed the literature review, contributed to the interpretation of the findings, wrote the manuscript and critically revised it. PM-B contributed to the interpretation of the findings and critically revised the manuscript. NP designed the study, supervised the writing of the manuscript, contributed to the interpretation of the findings and critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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CLINICAL TRIAL
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The Role of Traditional Chinese Formula Ding-Kun Pill (DKP) in Expected Poor Ovarian Response Women (POSEIDON Group 4) Undergoing *In Vitro* Fertilization-Embryo Transfer: A Multicenter, Randomized, Double-Blind, Placebo-Controlled Trial

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Objective: The primary objective of the study was to assess traditional Chinese formula DKP supplementation in terms of efficacy and safety on reproductive outcomes of expected poor ovarian responder (POR, POSEIDON Group 4) undergoing *in vitro* fertilization-embryo transfer (IVF-ET).

Design, Setting, and Participants: Women eligible for IVF-ET were invited to participate in this randomized, double-blind, placebo-controlled, superiority trial at academic fertility centers of ten public hospitals in Chinese Mainland. A total of 462 patients (35–44 years) equally divided between DKP and placebo groups with antral follicle count (AFC) <5 or anti-müllerian hormone (AMH) <1.2 ng/ml were randomized.

Interventions: All participants were given DKP or 7 g placebo twice daily on the previous menstrual cycle day 5 until oocyte retrieval, which took approximately 5 to 6 weeks.

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Main Outcome Measure: The primary outcome was the ongoing pregnancy defined as more than 20 gestational weeks of an intrauterine living fetus confirmed by pelvic ultrasonography.

Results: Demographic characteristics were equally distributed between the study populations. Intention-to-treat (ITT) analysis revealed that ongoing pregnancy rate (OPR) was not significantly different between DKP and placebo groups [26.4% (61/231) versus 24.2% (56/231); relative risk (RR) 1.09, 95% confidence interval (CI) 0.80 to 1.49, $P = 0.593$]. No significant differences between groups were observed for the secondary outcomes. The additional per protocol (PP) analysis was in line with ITT results: OPR in DKP group was 27.2% (61/224) versus 24.1% (55/228) in placebo group [RR 1.13, 95% CI (0.82 to 1.55), $P = 0.449$]. After subgroup analysis the findings concluded that POR population of 35–37 years had a significantly higher OPR after 5–6 weeks of oral DKP (41.8%, 33/79) versus placebo (25.4%, 18/71) [RR 1.65, 95% CI (1.02 to 2.65), $P = 0.034$, P for interaction = 0.028].

Conclusion: This well-designed randomized controlled trial (RCT) offers new high-quality evidence to supplement existing retrospective literature concerning DKP performance in expected PORs. DKP could be recommended as a safe and natural remedy for expected PORs (aged 35–37 years) who fulfill the POSEIDON group 4 criteria. However, additional interventional clinical studies are undoubtedly required to be conducted in the future to validate this hypothesis.

Clinical Trial Registration: www.chictr.org.cn, identifier ChiCTR1900026614.

Keywords: POSEIDON criteria, low prognosis, Ding-Kun Pill, traditional Chinese medicine, poor ovarian response, *in vitro* fertilization-embryo transfer

INTRODUCTION

The worldwide childbearing postponement is on the rise over the past few decades due to socioeconomic factors, such as accessibility to contraceptives, economic prosperity, improved education, and women's workforce engagement (1, 2). This delay contributes to increased average age of the first attempt at conception, a proportional increase in women's live births in their thirties, and higher pregnancy loss rates (3). The follow-through effect can be observed in the disproportionate use of assisted reproductive technology services among older women (4, 5). Besides, women are more vulnerable to decreased ovarian reserves (DOR) in their mid to late thirties associated with normal ovarian ageing, resulting in a growing number of older women having poor ovarian response (POR) during ovarian stimulation. This illustrates the necessity to devote significantly more attention to this women group undergoing *in vitro* fertilization-embryo transfer (IVF-ET) (6, 7).

The estimated POR prevalence ranges from 6 to 35%, which poses a severe challenge in assisted reproductive technology (8, 9). Moreover, large discrepancies in POR definition exist in preceding studies (10). This lack of uniformity resulted in the Bologna criteria in 2011, exemplifying the first significant attempt to establish specific POR definition standards (11). However, the Bologna criteria were questioned because of persistent heterogeneity among POR patients and the inability to provide management strategies (12, 13). Given the above-mentioned facts, more recent criteria, the POSEIDON classification, suggesting a new concept of low prognosis, was developed to provide a homogeneous and refined POR definition, resulting in significant heterogeneity reduction in Bologna POR population and individualized treatment promotion in these patients (14).

Among all POSEIDON groups, group 4 (age ≥35 years and AFC <5 or AMH <1.2 ng/ml) has been estimated to constitute 55% of patients (15). POSEIDON group 4 subpopulations have a considerably lower prognosis due to age-related increase in oocyte euploidy, leading to more aneuploid embryos and higher ET cancellation rates. Managing such patients is a daunting task; however, the treatment objective is to enhance the probability of producing at least one euploid blastocyst to be transferred to the individual patient. Although more evidence is required, this might be accomplished, possibly by adding adjuvant treatment to ovarian stimulation (OS) protocol or before OS initiation (15, 16). Regardless of the various pre-treatment strategies, comprising coenzyme Q10 and dehydroepiandrosterone (DHEA), insufficient evidence is found on the efficacy of these therapeutic agents to reverse low prognosis, particularly in women with advanced reproductive age or DOR (17–19).

DKP is one of the famous traditional Chinese medicine prescriptions that was first utilized during Emperor Qianlong's

reign of Qing dynasty (A.D. 1636–1912) as a unique formula in the emperor's harem, accompanied by exclusive utilization by the imperial court. The approved DKP formula empowered few companies to produce, such as Shanxi Guangyuyuan Traditional Chinese Medicine Co., Ltd., whose DKP was rated as National intangible cultural heritage by the State Council of the People's Republic of China in 2011. DKP components comprise ginseng, deer antler, safflower, angelica, scutellaria, rhizoma cyperi, ligustrazine, and other 30 precious Chinese herbal and animal orient medicine. Based on traditional Chinese medicine (TCM) theory, DKP has been deployed as a blood-activating and Qi-nourishing formula toward improving and curing several prevalent gynecological diseases, such as menstrual disorders, dysmenorrhea, menopausal syndrome and other physical symptoms (20). Meanwhile, modern pharmacological studies indicated that DKP could decrease blood viscosity, plasma viscosity and hematocrit, enhance inflammation and hypoxia, and promote mice's uterus development (21). In clinical practice of Chinese medicine, TCM pathogenesis in elderly women with low prognosis is mainly manifested by spleen and kidney deficiency, blood deficiency, and liver depression, consistent with TCM syndrome type of DKP. Moreover, no RCTs have investigated DKP supplementation effectiveness based on POSEIDON stratification in IVF cycles so far. In our previous prospective cohort study, we observed that older women with low prognosis following DKP pretreatment had more oocytes and embryos than those without DKP intervention. Although DKP group had a higher clinical pregnancy and ongoing pregnancy rates, yet the difference was not statistically significant (22).

As a consequence, such a well-designed randomized controlled trial devotes to investigating efficacy and safety of DKP on reproductive outcomes of IVF-ET in women with low prognosis who meet the POSEIDON group 4 criteria.

MATERIALS AND METHODS

Design and Participants

The study design was a multicenter, randomized, double-blinded, placebo-controlled, superiority trial with a 1:1 allocation to either DKP or placebo groups. Following the study approval by ethics committees of participating hospitals, all couples provided voluntary written informed consent prior to participation. A data and safety monitoring board have been established to manage the study. The study rationale and a detailed trial protocol have been published elsewhere previously (23). The current study followed the Consolidated Standards of Reporting Trials (CONSORT) reporting guideline.

Participants eligible for RCT underwent IVF/intracytoplasmic sperm injection (ICSI) cycles and fulfilled POSEIDON group 4 stratification based on the Bologna criteria. POSEIDON group 4 is known as ≥35 years old with poor pre-stimulation ovarian reserve parameters (AFC <5 or AMH <1.2 ng/ml) and with an expected poor ovarian response (fewer than four oocytes) after standard ovarian stimulation. The exclusion criteria were as follows: (i) Individuals with a Body Mass Index (BMI) ≥30 kg/m^2; (ii) Those using the natural cycle or mild stimulation for IVF/ICSI treatment; (iii) Those with a history of unilateral oophorectomy or recurrent pregnancy loss, defined as two or more spontaneous abortions; (iv) Acceptors of donated oocytes or performed either In vitro Maturation (IVM) or blastocyst biopsy for Preimplantation Genetic Diagnosis (PGD) or Preimplantation Genetic Testing for Aneuploidies (PGT-A); (v) Those previously diagnosed with congenital (e.g., mediastinal uterus and double uterus) or acquired (e.g., submucosal myoma and adenomyosis) uterine abnormalities; (vi) Patients with extremely advanced age (≥45 years old); and (vii) Presence of a non-surgically treated hydrosalpinx or endometrial polyp and an ovarian endometriosis cyst requiring surgery, during ovarian stimulation.

Randomization and Blinding

Eligible participants were invited to enroll in RCT by advertisement, and they were recruited from November 15, 2019 to July 7, 2020. A total of 462 couples were randomly allocated in four blocks into either DKP or placebo groups using a computerized random number generator (R 4.0.0, R Foundation for Statistical Computing, Vienna, Austria), ensuring a 1:1 allocation ratio. Therefore, each block resulted in allocating four patients to each group. A study staff generated the sequences and assigned the participants to DKP and placebo groups without taking part in intervention delivery, data collection, or data analysis. Participants were enrolled by staff without involving in randomization process. Both medications (DKP formula and placebo) were prepared with identical shape, taste, and smell.

Treatment Procedures

DKP Formula and Placebo Preparation

The decoction is generally prepared by boiling in water for hours. However, DKP (Lot No. 3271911068, Shanxi Guangyuyuan Traditional Chinese Medicine Co., Ltd, Shanxi, China) was prepared by adopting water-honeyed pill protocol according to Chinese Pharmacopoeia (ChP) 2015 Edition standard. The "DKP water-honeyed pill" standard is approved by the China Food and Drug Administration (CFDA). Each bottle is filled with 7 g DKP.

DKP is mainly composed of the following 30 medicinal herbs, including Radix Ginseng, Cornu Cervi Pantotrichum, Radix Angelicae sinensis, Radix Rehmanniae Preparata, Stigma Croci, Caulis Spatholobi, Radix Notoginseng, Radix Paeoniae Alba, Rhizoma Atractylodis Macrocephalae, Fructus Lycii, Radix Scutellariae, Rhizoma Cyperi, Fructus Leonuri, Rhizoma Ligustici Chuanxiong, Cornu Cervi Degelatinatum, Colla Corii Asini, Rhizoma Corydalis, Flos Carthami, Herba Leonuri, Faeces Togopteri, Poria, Radix Bupleuri, Radix Linderae, Fructus Amomi Villosi, Cortex Eucommiae, Rhizoma Zingiberis, Herba Asari, Radix Cyathulae, Cortex Cinnamomi, and Radix Glycyrrhizae.

The genuine medicinal materials are used in all kinds of traditional Chinese herbal and animal medicines, and specific purchasing locations are stipulated as township-level sales points where genuine medicinal materials are located and purchased in the same batch. The medicinal materials are processed according to requirements, and standard operating procedures are

formulated. The quality control results of DKP were consistent with Chinese Medicine Standards of State Food and Drug Administration (SFDA) (24, 25).

The placebo is provided by Shanxi Guangyuyuan Traditional Chinese Medicine Co. Ltd. (China) as a mixture of 55% starch and 45% caramel that were mixed, dried, crushed, and lumped together. The daily placebo doses are packed in individual bottles for easy consumption under ChP 2015 Edition standard, Good Manufacture Practice of Medical Products (GMP) standard. Patients in placebo group consume the same amount of placebo as treatment group. The placebo and Chinese medicines were used to make DKP identical in appearance, color, smell, taste, packaging, usage, and dosage (26). During placebo production, selecting condiments, colorants, and other excipients should be carefully carried out and strictly in accordance with Chinese Medicine Standards of SFDA.

Before ovarian stimulation, all participants can obtain DKP or 7 g placebo orally twice daily for approximately 5 to 6 weeks, from day 5 of the previous menstrual cycle until oocyte recovery.

Ovarian Stimulation Regimen

All participants started ovarian stimulation with a Gonadotropin-releasing hormone (GnRH) antagonist regimen on menstrual cycle day 2 or 3. GnRH antagonist (0.25 mg, cetrorelix; Merck Serono, Darmstadt, Germany) was administered subcutaneously at a daily dose of 0.25 mg when there is at least one follicle measuring ≥12 mm in mean diameter, with 150–300 IU/day of recombinant follicle-stimulating hormone (rFSH) (75IU, Puregon, MSD, Courbevoie, France; Gonal-F, Merck-Serono, Lyon, Italy) and recombinant luteinizing hormone (rLH) (75IU, Luveris®, Merck-Serono, Germany). Gonadotropin doses were determined based on individual patient's characteristics. Final oocyte maturation must be triggered when more than one leading follicle measuring 18 mm or greater are visible by ultrasound. Final oocyte maturation needed to be achieved by both 0.2 mg injection of GnRH agonist (0.1 mg, Triptoreline, Decapeptyl, Ipsen, France) and 250 μg of recombinant human chorionic gonadotropin (rhCG, 250 μg, Ovitrelle, Serono, France) (27). Oocyte retrieval was accomplished by transvaginal ultrasound-guided aspiration after 34–35 h.

Oocyte Retrieval and Embryo Culture

BD Falcon IVF medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was employed to collect oocytes and perform embryo culture. Incubation conditions were set at 6% CO_2, 5% O_2, and 37.0°C (C200 CO_2 Incubator, Labotect Labor-Technik-Göttingen GmbH, Göttingen, Germany). Cultures oocytes were inseminated for IVF or decumulated for ICSI.

Transfer of one or two high-quality embryos was performed on day 3 or 5, and the surplus was frozen on same days based on routine at various sites. All good quality embryos were cryopreserved *via* vitrification (CBS-ViT-HS, CryoBioSystem®, L'Aigle, France). Dimethylsulfoxide and ethylene glycol were used as cryoprotectants (Irvine Scientific Freeze Kit®, Irvine Scientific, Newtown Mount Kennedy, Ireland and Vitrification Kit 101, Cryotech®, Tokyo, Japan).

Endometrial Preparation and Embryo Transfer

For patients assigned to fresh embryo transfer, intramuscular progesterone at a daily dose of 60 mg was administered for luteal-phase support, beginning on oocyte retrieval day until 8–10 weeks after conception. For patients assigned to frozen–thawed embryo transfer (FET), no luteal-phase support was administered after oocyte retrieval, and day-3 embryos or day-5 blastocysts were cryopreserved for later transfer.

Artificial endometrial preparation consisted of sequential administration of E_2 valerate and intramuscular progesterone. A total of 2 mg E_2 valerate were administered twice daily for 6–8 days, and the dose was later adjusted based on endometrial thickness measured by vaginal ultrasonography. For endometrial thickness ≥7 mm, intramuscular progesterone 60 mg was initiated, while for endometrial thickness <7 mm, the patients continued taking oral E_2 until the endometrium attained the required threshold. On day 4 or 6 of progesterone regimen, one or two day-3 frozen embryos or day-5 blastocysts were thawed and transferred. Ultrasound-guided soft catheter embryo transfers were performed. Once pregnancy was confirmed 14 days after FET, the luteal-phase support with estradiol valerate and intramuscular progesterone for endometrial preparation continued until 8–10 weeks of gestation.

Study Outcomes

This work's primary outcome was ongoing pregnancy rate per randomized patient, which also included natural pregnancies. We defined ongoing pregnancy as a detectable fetal heartbeat after 20 weeks of gestation. Ongoing pregnancy rate was recorded per randomized patient, started stimulation, oocyte retrieval, and embryo transfer. Secondary outcomes were positive pregnancy rates (biochemical pregnancies), embryo implantation rates, clinical pregnancy rates, ectopic pregnancy rates, pregnancy loss rates, and twin pregnancies, including women admitted to hospital for abnormal pregnancies. Definitions for secondary outcomes are provided in **Supplementary Table 2**.

Statistical Analysis

We designed the trial as a superiority study using PASS software version 11.0 (NCSS, LLC. Kaysville, Utah, USA) to calculate sample size. Sample size calculation indicated that at least 203 patients in each group were required to have a 90% power at a significance level of 0.05 to detect an absolute difference of 15% in the ongoing pregnancy rate with DKP supplementation, with an estimated rate of 25% in Placebo group. The effect size of 15% was based on existing scarce literature. Our previous study found a difference of 14.4% in the ongoing pregnancy rate for DKP group compared with non-DKP one (22). Therefore, the effect size of 15% was based on these limited numbers; however, the trial plans to include 462 participants, with 231 in each arm, to account for an expected 10% loss to follow-up.

We used the intention-to-treat principle for the primary statistical analysis. Primary and secondary outcomes were assessed by comparing the outcome after the first embryo transfer. All women were accounted for in the group to which they were randomized, regardless of whether they received the

prespecified treatment. We included all women who adhered strictly to the study protocol in a *post hoc* per protocol and subgroup analyses (**Figure 1** and **Supplementary Table 1**). We determined ongoing pregnancy rate, and relative risk was used to describe the difference. We compared continuous data utilizing a Student t-test or a Wilcoxon rank-sum test, and the results are given as mean (SD, standard deviation) or median (IQR, interquartile range). Categorical data were assessed using chi-square analysis and Fisher's exact test for expected frequencies less than 5. A two-sided P value of <0.05 was considered to indicate statistical significance. All analyses were performed using SPSS version 26.0 and R statistical package version 4.0.0.

RESULTS

Study Patients

The baseline demographics and clinical characteristics of patients were comparable between the study groups (**Table 1**). A total of 10 patients deviated from the protocol, including seven of 231 (3.0%) in DKP group and three of 231 (1.3%) in placebo group (**Figure 2**). Of these women, 453 had oocytes retrieved: 225 (97.4%) in DKP group and 228 (98.7%) placebo group. After ovarian stimulation, four (1.7%) in DKP group and eight (3.5%) in placebo group had no oocytes retrieved (**Table 2**). Additionally, 14 women (6.1%) in DKP group and nine (3.9%) in placebo group did not have an embryo available for transfer (**Table 2**). Two (0.9%) women in placebo group did not have a blastocyst for transfer, and one woman (0.4%) in DKP group had all oocytes frozen (**Table 2**).

COS and IVF Characteristics

Table 2 shows the characteristics of enrolled women according to COS and IVF procedures. No significant differences were found in most COS and IVF outcomes between DKP and placebo groups. Conversely, median number of high-quality blastocysts was significantly higher in DKP group (2.0, IQR 1.0) compared with placebo group (1.0, IQR 0; P = 0.014).

Ongoing Pregnancy and Secondary Outcomes

The primary analysis was performed according to the intention-to-treat principle. In DKP group, 61 of 223 women (26.4%) had an ongoing pregnancy compared with 56 of 231 (24.2%) in placebo group, for a relative risk (RR) of 1.09 [95% confidence interval (CI), 0.80 to 1.49; P = 0.593; **Table 3**]. No significant difference was found in clinical pregnancy rate between DKP and placebo groups [77 of 231 (33.3%) and 70 of 231 (30.3%), respectively; RR 1.10, 95% CI 0.84 to 1.44, P = 0.484; **Table 3**]. One woman in placebo group conceived naturally before oocyte retrieval (**Figure 2**). No significant difference was present in embryo implantation rate, ectopic pregnancy rate, pregnancy loss rate, and twin pregnancies between the two groups.

The frequency of positive pregnancy, clinical pregnancy, and ongoing pregnancy per embryo transfer was not significantly different between DKP and placebo groups. The ongoing pregnancy rate per embryo transfer was 29.6% (61/206) versus 26.3% (55/209) in DKP and placebo groups, respectively (RR 1.13, 95% CI 0.83 to 1.53, P = 0.454; **Table 3**). The per-protocol analysis results were consistent with intention-to-treat analysis results, as displayed in **Supplementary Table 1**.

Subgroups	Ding-Kun Pill group		Placebo group		Relative Risk (95%CI)	P value	P value for interaction
	Total No.	Events (%)	Total No.	Events (%)			
Total	206	61 (29.6)	209	55 (26.3)	1.13 (0.83 to 1.53)	0.454	
Female age at oocyte retrieval							0.028
< 37 yrs.	79	33 (41.8)	71	18 (25.4)	1.65 (1.02 to 2.65)	0.034	
≥ 37 yrs.	127	28 (22.0)	138	37 (26.8)	0.82 (0.54 to 1.26)	0.368	
Body mass index (BMI)							0.394
< 24 kg/m^2	128	35 (27.3)	137	37 (27.0)	1.01 (0.68 to 1.50)	0.951	
≥ 24 kg/m^2	78	26 (33.3)	72	18 (25.0)	1.33 (0.80 to 2.22)	0.263	
FSH on menstrual cycle days 2-3							0.147
≤ 10 mIU/ml	129	44 (34.1)	124	32 (25.8)	1.32 (0.90 to 1.94)	0.15	
> 10 mIU/ml	77	17 (22.1)	85	23 (27.1)	0.82 (0.47 to 1.41)	0.463	
Embryo transfer stage							0.402
Cleavage stage	178	48 (27.0)	191	45 (23.6)	1.15 (0.81 to 1.63)	0.451	
Blastocyst stage	28	13 (46.4)	18	10 (55.6)	0.84 (0.47 to 1.48)	0.546	
No of embryos transferred							0.624
Single embryo transfer	81	18 (22.2)	82	18 (22.0)	1.01 (0.57 to 1.80)	0.967	
Double embryo transfer	125	43 (34.4)	127	37 (29.1)	1.18 (0.82 to 1.70)	0.369	
Embryo transfer protocol							0.234
Fresh embryo transfer	106	32 (30.2)	123	38 (30.9)	0.98 (0.66 to 1.45)	0.908	
Frozen-thawed embryo transfer	100	29 (29.0)	86	17 (19.8)	1.47 (0.87 to 2.48)	0.146	
Method of fertilization							0.314
IVF	146	39 (26.7)	155	41 (26.5)	1.01 (0.69 to 1.47)	0.959	
ICSI	60	22 (36.7)	54	14 (25.9)	1.41 (0.81 to 2.48)	0.218	
Top-quality embryos transferred							0.177
Yes	77	29 (37.7)	67	21 (31.3)	1.20 (0.76 to 1.90)	0.427	
No	129	32 (24.8)	142	34 (23.9)	1.04 (0.68 to 1.58)	0.869	

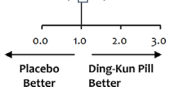

FIGURE 1 | Subgroup analysis of ongoing pregnancy rate per embryo transfer for women in Ding-Kun Pill and placebo groups. (FSH, follicle stimulating hormone; IVF, *in vitro* fertilization; ICSI, intracytoplasmic sperm injection).

TABLE 1 | Participants' baseline characteristics on menstrual cycle days 2–3.

Characteristics	Ding-Kun Pill group (n = 231)	Placebo group (n = 231)	P value
Age at inclusion (years; mean (SD)):	37.9 (2.3)	37.8 (2.2)	0.771
Age ≥37	147 (63.6)	155 (67.1)	0.434
Age ≥40	67 (29.0)	58 (25.1)	0.346
Body mass index (kg/m^2; mean (SD)) ⁂	23.2 (3.0)	23.0 (2.9)	0.372
Duration of infertility (years; mean (IQR))	3.3 (4.0)	4.0 (4.0)	0.307
Nulliparous	68 (29.4)	82 (35.5)	0.164
Primary cause of infertility:			0.816
Tubal factor	168 (72.7)	159 (68.8)	
Male factor	52 (22.5)	59 (25.5)	
Tubal + Male factor	7 (3.0)	9 (3.9)	
Unexplained infertility	4 (1.7)	4 (1.7)	
AMH (ng/ml; median (IQR))	0.9 (0.6)	0.9 (0.6)	0.677
Total AFC (mean (IQR))	5.0 (2.0)	5.0 (2.0)	0.561
FSH (mIU/ml; mean (IQR)) ⁋	9.0 (5.1)	9.0 (5.2)	0.795
LH (mIU/ml; mean (IQR)) ⁋	4.2 (3.3)	4.6 (3.0)	0.128
Estradiol (pg/ml; mean (IQR)) ⁋	46.3 (30.0)	50.1 (30.0)	0.367

In any of the baseline characteristics, no significant differences between groups (P <0.05) were observed. AMH, anti-müllerian hormone; AFC, antral follicle count; FSH, follicle stimulating hormone; LH, luteinizing hormone; IQR, interquartile range; SD, standard deviation.

⁂*Body mass index is weight (kg) divided by height squared (m^2).*

⁋*FSH was missing for two women in placebo group. LH was missing for one woman in Ding-Kun Pill group and for three women in placebo group. Estradiol was missing for one woman in Ding-Kun Pill group and for three women in placebo group.*

Data are presented as numbers (%) unless otherwise noted.

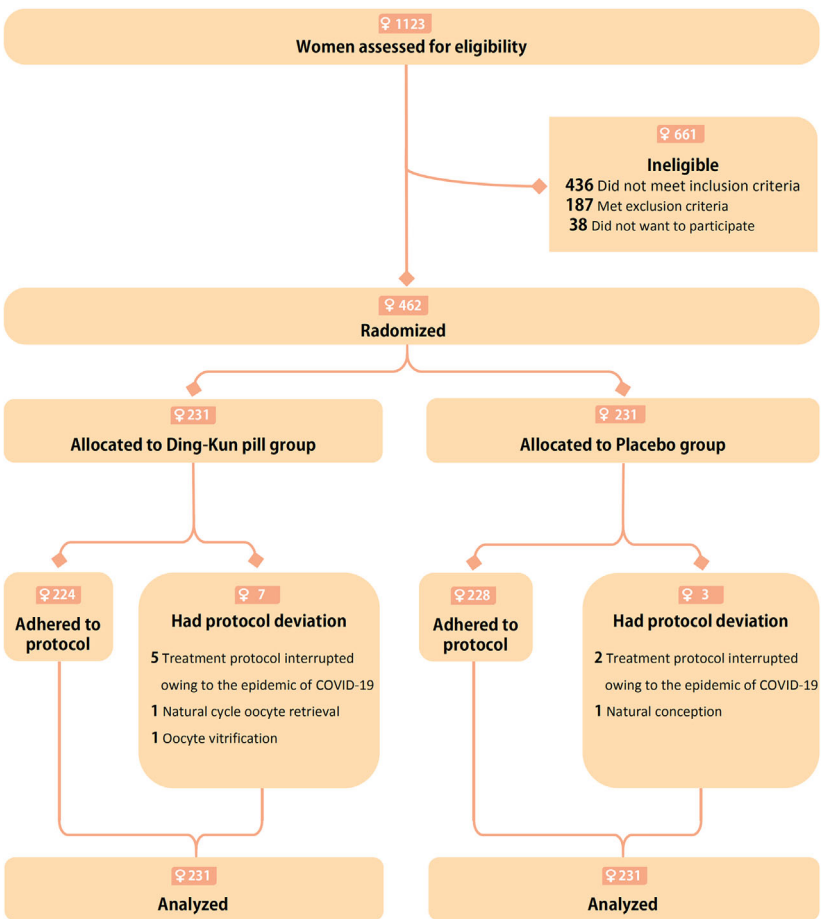

FIGURE 2 | Flow chart depicting the randomized assignment of women to the Ding-Kun Pill and Placebo groups, exclusions, and protocol deviations.

TABLE 2 | Controlled ovarian stimulation and *in vitro* fertilization-embryo transfer characteristics in study population.

Characteristics	Ding-Kun Pill group (n = 231)	Placebo group (n = 231)	P value
No. of days of COS (mean (SD))	9.6 (2.4)	9.5 (2.4)	0.504
Total gonadotrophin dose administered (IU; mean (IQR))	2,100 (2,325)	2,025 (1,350)	0.269
Estradiol on hCG trigger day (pg/ml; mean (IQR))	1,214.6 (857.3)	1,281 (999)	0.465
Progesterone on hCG trigger day (ng/ml; mean (IQR))	0.6 (0.6)	0.7 (0.5)	0.427
Method of fertilization:			0.435
IVF	162/224 (72.3)	170/225 (75.6)	
ICSI	62/224 (27.7)	55/225 (24.4)	
No. of oocytes retrieved (median (IQR))	6.0 (3.0)	6.5 (4.0)	0.855
No. of two PN oocytes (fertilized; median (IQR)) †	5.0 (3.0)	4.0 (5.0)	0.383
No. of two PN cleavage zygotes (median (IQR))	5.0 (4.0)	4.0 (4.0)	0.279
No. of embryos available for transfer (median (IQR))	3.0 (3.0)	4.0 (3.0)	0.265
No. of high-quality day 3 embryos (median (IQR)) ‖	2.0 (2.0)	2.0 (2.0)	0.355
No. of high-quality blastocysts (median (IQR)) ‡	2.0 (1.0)	1.0 (0)	0.014
No. of embryos transferred (mean (IQR)):	2.0 (1.0)	2.0 (1.0)	0.983
Single embryo transfer	81/206 (39.3)	82/209 (39.2)	0.986
Double embryo transfer	125/206 (60.7)	127/209 (60.8)	0.986
Embryo transfer stage:			0.106
Cleavage stage	178/206 (86.4)	191/209 (91.4)	
Blastocyst stage	28/206 (13.6)	18/209 (8.6)	
Fresh embryo transfer	102/202 (50.5)	121/207 (58.5)	0.106
Endometrial thickness on hCG trigger day (mm; mean (SD))	9.8 (1.8)	9.9 (2.3)	0.839
Frozen-thawed embryo transfer	100/202 (49.5)	86/207 (41.5)	0.106
Endometrial thickness prior to FET (mm; mean (SD)) §	9.1 (1.4)	9.2 (1.7)	0.877
No. of women with no oocytes retrieved after COS	4/225 (1.8)	8/228 (3.5)	0.264
No. of women with no embryo transfer after aspiration:			0.169
No blastocyst development	0/224	2/225	
No day-3 embryo available for transfer	14/224	9/225	
Oocyte vitrification	1/224	0/225	

COS, controlled ovarian stimulation; hCG, human chorionic gonadotropin; IVF, in vitro *fertilization; ICSI, intracytoplasmic sperm injection; IQR, interquartile range; SD, standard deviation.*
†Two distinct pronuclei defined by four cells, a maximum of 10% fragmentation, and no multinucleation.
‖Typically, a good, normally growing day 3 embryos will contain between six and 10 cells.
‡Defined as Gardner score 3BB or higher.
§Programmed cycle defined by administration of both estradiol and progesterone.
Data are number/total number or number (%) unless stated otherwise.

Subgroup Analysis of Ongoing Pregnancy Rate Per Embryo Transfer

The results were similar across most subgroups; nevertheless, the advantage of DKP supplementation tended to be more pronounced among patients younger than 37 years than in elderly patients between 37 and 44 years [33/79 (41.8%) versus 18/71 (25.4%); RR 1.65, 95 CI 1.02 to 2.65; P = 0.034; P = 0.028 for interaction] (**Figure 1** and **Supplementary Table 3**).

Safety Assessment

No adverse events occurred in either DKP group versus placebo group. Safety indicators of complete blood cell count and liver and kidney function before and after treatment were within reasonable limits in both groups.

DISCUSSION

To the best of our knowledge, this is the largest RCT performed to evaluate DKP supplementation effects on reproductive outcomes in patients undergoing IVF-ET who fulfilled POSEIDON group 4 criteria. In this multicenter, randomized controlled trial, we found no significant differences in ongoing pregnancy and clinical pregnancy rates between DKP and placebo groups. However, DKP supplementation resulted in a significantly higher number of high-quality blastocysts compared with placebo group. Besides, the benefit of utilizing DKP seemed to be more apparent in patients below 37 years than in ones between 37 and 44 years.

According to TCM principle, POR belongs to categories of 'infertility', 'hypomenorrhea', 'amenorrhea' and 'menopausal syndrome'. In TCM, POR is associated with spleen and kidney deficiency, liver depression and blood vacuity (28, 29). The Yellow Emperors Internal Classic, a famous book published more than 2,000 years ago, states that females' basic physiological processes are linked to kidneys. Besides, stagnation of liver and spleen are thought to be responsible for POR (30). Accordingly, improving the physical condition of kidney, liver and spleen may improve POR. The present study investigated DKP effect on POR who meet the POSEIDON group 4 stratification. The DKP is derived from 'Si Wu' decoction, 'Si Jun Zi' decoction, and 'Chaihu Shugan' powder and includes more than 30 types of Chinese herbal and animal medicines. In TCM, this mixture is assumed to tonify the kidney, invigorate blood circulation, smooth the liver and invigorate the spleen. As a result, it was hypothesized that DKP may be beneficial for POR patients.

TABLE 3 | Reproductive outcomes for women in Ding-Kun Pill and placebo groups (intention-to-treat analysis).

Outcomes	Ding-Kun Pill group (n = 231)	Placebo group (n = 231)	Relative risk (95% CI)	P value
Primary outcome				
Ongoing pregnancy *				
Ongoing pregnancy rate/No. of randomised women	61/231 (26.4)	56/231 (24.2)	1.09 (0.80 to 1.49)	0.593
Ongoing pregnancy rate/No. of women who started stimulation	61/225 (27.1)	55/228 (24.1)	1.12 (0.82 to 1.54)	0.466
Ongoing pregnancy rate/No. of oocyte retrievals	61/225 (27.1)	55/225 (24.4)	1.11 (0.81 to 1.52)	0.518
Ongoing pregnancy rate/No. of embryo transfers	61/206 (29.6)	55/209 (26.3)	1.13 (0.83 to 1.53)	0.454
Secondary outcomes				
Clinical pregnancy				
Clinical pregnancy rate/No. of randomised women	77/231 (33.3)	70/231 (30.3)	1.10 (0.84 to 1.44)	0.484
Clinical pregnancy rate/No. of women who started stimulation	77/225 (34.2)	69/228 (30.3)	1.13 (0.87 to 1.48)	0.367
Clinical pregnancy rate/No. of oocyte retrievals	77/225 (34.2)	69/225 (30.7)	1.12 (0.85 to 1.46)	0.421
Clinical pregnancy rate/No. of embryo transfers	77/206 (37.4)	69/209 (33.0)	1.21 (0.81 to 1.81)	0.352
Positive pregnancy †				
Positive pregnancy rate/No. of randomised women	85/231 (36.8)	82/231 (35.5)	1.04 (0.81 to 1.32)	0.771
Positive pregnancy rate/No. of women who started stimulation	85/225 (37.8)	81/228 (35.5)	1.06 (0.84 to 1.36)	0.619
Positive pregnancy rate/No. of oocyte retrievals	85/225 (37.7)	81/225 (36.0)	1.05 (0.74 to 1.58)	0.696
Positive pregnancy rate/No. of embryo transfers	85/206 (41.3)	81/209 (38.8)	1.07 (0.84 to 1.35)	0.602
Pregnancy loss rate ‡	38/85 (44.7)	36/82 (43.9)	1.02 (0.73 to 1.43)	0.917
Pregnancy loss ≤12 weeks of gestation	37/85 (43.5)	34/82 (41.5)	1.05 (0.74 to 1.50)	0.787
Pregnancy loss >12 weeks of gestation	1/85 (1.2)	2/82 (2.4)	0.48 (0.05 to 5.22)	0.616
Ectopic pregnancies ‡	0/85 (0)	1/82 (1.2)	–	0.491
Embryo implantation rate (median (IQR)) ‖	0 (0.5)	0 (0.5)	–	0.500
Twin pregnancies	5/77 (6.5)	11/70 (15.7)	0.41 (0.15 to 1.13)	0.073

All analyses by intention to treat.

**Ongoing pregnancy was defined as a detectable fetal heart beat after 20 weeks of gestation.*

†Positive pregnancy (biochemical pregnancy), i.e. serum β-hCG level ≥10 mIU/ml.

‡Denominator defined as number of positive β-hCG values (≥10 IU/ml) in each group.

‖Embryo implantation rate was defined as the number of intrauterine gestational sacs observed divided by the number of embryos transferred.

Data are number/total number (%) of women unless stated otherwise.

Actually, DKP has been used in polycystic ovarian syndrome (PCOS) with concomitant ovulatory defects, insulin resistance, and menstrual abnormalities, although clinical trials utilizing DKP in POR population are quite scarce (31, 32). Two randomized controlled trials (RCTs) have recently investigated DKP supplementation's role in POR therapy (30, 33). Xie discovered that using DKP and clomiphene in treating patients with reduced ovarian reserve substantially raised FSH, AFC, AMH, and estradiol levels as opposed to clomiphene alone. Moreover, their life quality, ovulation rate, and clinical pregnancy rate all increased dramatically ($P < 0.01$) (33). However, several variables render it impossible to reliably randomize participants, and since this was performed in an outpatient clinic, the results made cannot be verified and have little particular scientific value for women undergoing IVF-ET. In patients with a poor response to OS, Wei and his colleagues evaluated the impact of DKP and micro ovarian stimulation on clinical outcomes. The findings showed that compared to non-DKP group, DKP significantly increased estradiol concentration and endometrial thickness on hCG trigger day, decreased Gn dose, duration and cycle cancellation rate, and increased numbers of oocytes retrieved, high-quality embryos, embryo implantation rate, and clinical pregnancy rate (30). The study described above had clear randomization but not blinding and allocation concealment; meanwhile, the participants' recruitment process was unclear, and the sample size estimate was uncertain. Additionally, the inclusion criteria were introduced by the 2012 Bologna Consensus, but eligible participants were not stringent, and there was considerable variability between populations (30). Fortunately, DKP pretreatment promotes ovarian sensitivity to exogenous gonadotropins in POR patients, but this may contribute to greater oocyte developmental capacity, greater endometrial receptivity, and a higher clinical pregnancy rate, all of which allows DKP to have certain benefit.

The detailed molecular mechanisms of the effect of DKP on oocytes, cumulus cells, and granulosa cells are still unclear. A recent experimental study found that DKP can effectively activate the implantation rate of delayed embryo implantation mouse model by regulating the genes related to 'endometrium-embryo interface' (34). Moreover, according to Ma et al., it was demonstrated that DKP was able to increase ovarian reserves through inhibiting PI3K/AKT/mTOR signaling pathway, leading to suppression of primordial follicle activity and a reduction in levels of apoptosis of early growing follicles (35). All in all, these findings demonstrate the potentially beneficial role of DKP in treating DOR or POR. However, further studies are required to explore the molecular mechanisms underlying DKP actions.

Our trial is the first and largest multicenter randomized placebo-controlled trial to date to investigate DKP supplementation impact in POR patients. Moreover, we performed randomization at baseline on menstrual cycle day 2 or 3. This approach ensured minimal selection bias in the women

included in our study and not only those with more oocytes retrieved after ovarian stimulation. Furthermore, low prognosis patients were classified by POSEIDON groups which significantly reduced the heterogeneity identified in Bologna POR population. The same dosage of DKP or placebo was used in all patients treated. Our study has some limitations. Based on our previous study, even though we have prolonged the DKP period of intervention in patients with POR, we must conduct more studies on the optimum DKP treatment duration in the future (22). Moreover, the superiority study design had the power to detect a 15% difference in ongoing pregnancy rate between the two groups; therefore, smaller but clinically important differences might be overlooked.

CONCLUSIONS

In conclusion, DKP pretreatment in an IVF/ICSI cycle can improve the number of high-quality blastocysts in patients who accomplish the POSEIDON group 4 criteria. Moreover, DKP supplementation may raise OPR, especially in patients younger than 37 years. However, larger, well-designed interventional studies are required to further demonstrate the clinical relevance of DKP on improving reproductive outcomes for these subpopulations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Health Authorities and Ethics Committees of the Affiliated Hospital of Shandong University of TCM. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Z-GS had full access to all data in the study and takes responsibility for data integrity and data analysis accuracy. Concept and design: J-YS and Z-GS. Acquisition, analysis, or interpretation of data: D-DG, X-LC, and SX. Drafting of the manuscript: J-YS. Critical revision of the manuscript for important intellectual content: Y-HC, Y-LT, X-FL, and H-PL. Statistical analysis: F-XW, BZ, L-HX, LZ, and X-HH. Supervision: Z-GS and Y-LT. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.675997/full#supplementary-material

Supplementary Figure 1 | Dingkun Pill, a traditional Chinese medicine with a long history.

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MINI REVIEW
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Interplay Between mTOR and Hippo Signaling in the Ovary: Clinical Choice Guidance Between Different Gonadotropin Preparations for Better IVF

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One of the most widely used types of assisted reproduction technology is the *in vitro* fertilization (IVF), in which women undergo controlled ovarian stimulation through the administration of the appropriate hormones to produce as many mature follicles, as possible. The most common hormone combination is the co-administration of gonadotropin-releasing hormone (GnRH) analogues with recombinant or urinary-derived follicle-stimulating hormone (FSH). In the last few years, scientists have begun to explore the effect that different gonadotropin preparations have on granulosa cells' maturation and apoptosis, aiming to identify new predictive markers of oocyte quality and successful fertilization. Two major pathways that control the ovarian development, as well as the oocyte–granulosa cell communication and the follicular growth, are the PI3K/Akt/mTOR and the Hippo signaling. The purpose of this article is to briefly review the current knowledge about the effects that the different gonadotropins, used for ovulation induction, may exert in the biology of granulosa cells, focusing on the importance of these two pathways, which are crucial for follicular maturation. We believe that a better understanding of the influence that the various ovarian stimulation protocols have on these critical molecular cascades will be invaluable in choosing the best approach for a given patient, thereby avoiding cancelled cycles, reducing frustration and potential treatment-related complications, and increasing the pregnancy rate. Moreover, individualizing the treatment plan will help clinicians to better coordinate assisted reproductive technology (ART) programs, discuss the specific options with the couples undergoing IVF, and alleviate stress, thus making the IVF experience easier.

Keywords: granulosa cells, r-hFSH, r-hLH, HP-hMG, ovarian stimulation, PI3K/mTOR/Akt, Hippo

INTRODUCTION

The theory that FSH and luteinizing hormone (LH) are both required for the complete stimulation of follicular maturation and steroidogenesis was put forward 60 years ago from the Swedish scientist Bengt Falck (1). This idea was the basis for the stimulation of both hormonal systems for optimal follicular growth and maturation in IVF programs. Nowadays, ovarian stimulation during IVF includes the co-administration of GnRH analogues with the gonadotropins FSH, LH, and human chorionic gonadotropin (hCG).

A major drawback in IVF approaches is that the percentage of successful pregnancies is still low – approximately 27% pregnancies per IVF treatment in Europe (2, 3). Moreover, there is still a need for interventions to improve the initial recruitment and later survival of follicles to ensure good quality oocytes in healthy women, as well as in patients with poor ovarian response (POR), primary ovarian insufficiency (POI), or polycystic ovary syndrome (PCOS). Many studies compare the effects of different FSH-containing gonadotropin preparations in ovarian stimulation and IVF cycle outcomes, namely highly purified urinary human menopausal gonadotropin (HP-hMG) containing both FSH and LH activity, and recombinant human FSH (r-hFSH) alone or in combination with recombinant human LH (r-hLH). However, in most cases, the results are contradictory and inconclusive, and have led to controversial interpretations regarding the effectiveness of these gonadotropin regimens on follicular growth, antral follicle count, total oocytes retrieved, 2 pronuclear stage (2PN) oocytes, number of embryos, clinical pregnancy, and live birth rates in IVF (4–9). A pioneering study, a few years ago, demonstrated that the r-hFSH/r-hLH combination was more effective compared to HP-hMG, when the number of retrieved oocytes was high, also with regard to pregnancy rate per embryo transfer (10). Importantly however, a critical component of the stimulation regimens in IVF is the administration of a GnRH analogue, either agonist or antagonist to control the premature LH surge (11). Accordingly, an increasing number of studies reveal that the efficacy and the clinical outcomes of the different gonadotropin regimens appear to be dependent also, on the GnRH protocol used (9, 12–18). It is well known that the GnRH analogues can activate specific signal transduction pathways leading to distinct biological responses (19). Apparently, these treatments can alter the hormonal milieu, thereby favoring or hindering embryo quality and pregnancy rate (20). It is pertinent to note that FSH through binding to its cognate receptor FSHR (21), regulates the proliferation and differentiation of granulosa cells and prepares them to respond to gonadotropins and other endocrine signals, in order to undergo their final maturation. FSH is a glycoprotein, and it was recently shown that the hypo-glycosylated forms might be more efficient in promoting follicular growth and supporting granulosa cell survival *in vivo*, possibly by increasing serum estradiol levels (22). Interestingly, young women express partially glycosylated FSH whereas postmenopausal women express mainly the fully glycosylated form (23, 24), and this might influence both the biochemical properties and the efficacy of the various FSH preparations (25). This issue has been thoroughly discussed in a Delphi Consensus study recently (26).

THE DIFFERENTIAL EFFECTS OF GONADOTROPIN TREATMENTS ON OOCYTE – GRANULOSA CELL COMMUNICATION AND FOLLICULAR MATURATION

Considering the vital role of granulosa cells in oocyte and follicle maturation, scientists have sought to investigate the influence of gonadotropin treatment on granulosa gene expression profiles. For example, the administration of r-hFSH, in comparison to HP-hMG (27) has been associated with higher expression of LH receptors and enzymes involved in the biosynthesis of steroids, and with lower mRNA levels of the FSH receptors in the granulosa cells (28). The presence of the FSH ligand (in cultured rat and bovine granulosa cells) leads to follicular activation and steroidogenesis, through the action of the highly conserved phosphoinositide-3 kinase (PI3K)/Akt/mammalian (or mechanistic) target of rapamycin (mTOR) and Hippo signaling pathways (29–31). The dysregulation of these pathways leads to increased apoptosis in ovarian cells (32, 33). Importantly, the incidence of apoptosis in granulosa cells has been linked to the quality of the oocytes and to the pregnancy outcome (34–36). There is some evidence indicating that the administration of HP-hMG increases the apoptosis of cumulus cells compared to r-hFSH or urinary FSH (37), and a recent study showed that high doses of r-hFSH suppress the apoptosis of granulosa cells in patients with endometriosis undergoing IVF (38). Therefore, researchers are currently exploring the consequences of the different protocols of gonadotropin ovarian stimulation on the apoptosis rate of granulosa cells (35, 39). However, in the ART clinical setting, more upstream effectors need to be considered since follicular growth is a dynamic and continuous process, characterized by a tightly regulated equilibrium between apoptosis and cell proliferation. For example, recently, it was elegantly shown that the FSH receptor synergizes with the G protein-coupled estrogen receptor (GPER), hence reprogramming FSH-induced death signals to proliferative stimuli that are important for nourishing oocyte survival (40). Heterodimerization of GPER with FSHR in granulosa cells switches the signaling mode from cAMP to pAKT activation, thereby positively affecting follicle maturation, and appears to correlate with the FSH responsiveness of patients undergoing IVF. This is particularly interesting, in light of evidence showing that estrogen can regulate Hippo signaling *via* GPER in breast tissue (41, 42). Accordingly, it might be more insightful to investigate the effects of the different gonadotropin preparations on the maturation of granulosa cells and the oocyte quality by monitoring the activity of the PI3K/Akt/mTOR and Hippo signaling cascades.

Although there are no studies yet comparing the effect of different gonadotropins on the Hippo pathway, there are data showing that r-hFSH and HP-hMG can differentially modulate the activities of the PI3K/Akt/mTOR signaling. For example, Ji et al., 2020 (43) using a GnRH antagonist protocol, observed that

HP-hMG resulted in significantly higher insulin-like growth factor-1 (IGF-1) levels compared to r-hFSH on the day of oocyte retrieval, an effect that has been associated with better oocyte quality and pregnancy rate (44, 45). Interestingly, this was not the case in earlier studies when a GnRH agonist protocol had been employed (20, 46). The insulin/IGF-1 signaling pathway regulates the PI3K/mTOR/p70S6K cascade which as mentioned above plays an essential role in the FSH-mediated development of granulosa cells (30, 47, 48). This is important, also in light of recent findings showing that the hypo-glycosylated form of FSH, which is less abundant in the pituitary of postmenopausal women, activates more efficiently the PI3K/mTOR/p70S6K signaling (22).

Adding to the complexity of these interactions is the fact that there are many other signaling cues converging on both pathways. For example, other growth factors in addition to insulin/IGF-1, such as EGF, PDGF or VEGF are potent regulators of the PI3k/Akt/mTOR signaling in the follicles (49). Moreover, steroid hormones, like androgens which are the precursors for estrogen production, and known to stimulate granulosa and theca cell proliferation and to promote early antral follicle growth, can also regulate the expression of both FSH and IGF-1 receptor genes (50, 51). Furthermore, complex disorders such as the PCOS syndrome can affect the activation of both mTOR and Hippo signaling pathways. The development of PCOS has been associated with Hippo disruption and YAP overactivation leading to multiple early antral follicles and theca hyperplasia (49, 52). In addition, the expression of mTOR is elevated in a DHEA-treated PCOS animal model that could lead to insulin resistance, which is a characteristic of the PCOS phenotype (53). Other pathological conditions, such as endometriosis and ovarian cancer can exert an impact on the mTOR pathway by altering the expression of its targets (54). Scientists have also noticed increased expression of YAP protein in mouse models with endometriosis whereas in mice treated with YAP inhibitors the endometriotic lesions were significantly decreased (55). Notably, the activation of mTOR pathway plays a fundamental role in the development of many autoimmune disorders (56), whereas Hippo signaling prevents autoimmunity and tissue damage (57, 58). In addition, vitamin D deficiency decreases mTOR activation in rat models (59) and human uterine fibroid cells (60). These are conditions that can influence the IVF outcomes (61–64). Future studies addressing the effects of the various gonadotropin combinations on the PI3k/Akt/mTOR and Hippo pathways in physiological conditions (including ageing) and disease states, are expected to increase our understanding of follicle development and develop personalized treatment plans that will help clinician's decision and improve the success rate of IVF.

THE INTERPLAY BETWEEN PI3K/AKT/mTOR AXIS AND HIPPO PATHWAY IN FOLLICULAR DEVELOPMENT

The PI3K/Akt/mTOR axis is a key regulator of survival that fosters the processes of proliferation and differentiation, and inhibits apoptosis and autophagy (65, 66). The activation of this pathway is crucial for granulosa cell proliferation and follicular growth, especially during the primordial follicle development (67). Recent work from our lab revealed that the controlled pharmacological inhibition of the mTOR pathway in a rat experimental model can increase the number of competent primordial follicles while reducing atresia. Specifically, we showed that the follicles preserve their competence to resume growth two weeks after mTOR reactivation (68). Consistent with this, factors like Tsc1/2 and PTEN, which negatively regulate mTORC1, are capable to maintain the dormancy state of primordial follicles (69). Deregulation of these inhibitors leads to overactivation of the mTOR pathway that is linked to pathological situations where the entire pool of primordial follicles matures simultaneously resulting in an accelerated loss of primordial follicles and premature ovarian failure (POF) (70, 71). Over-activation of the mTOR pathway has been also associated with the emergence of PCOS and ovarian cancer (72). Importantly, however, there are no studies yet comparing the activation of mTOR pathway on granulosa cells obtained from IVF patients undergoing different protocols of gonadotropin stimulation.

Recent studies indicate that the Hippo signaling plays an instrumental role in the regulation of follicular growth. This pathway responds to mechanotransduction signals in order to maintain organ size through regulating cell proliferation and apoptosis (73, 74). The central components of the Hippo pathway are the kinases Mst1/2 and Lats1/2 which lead to the inactivation of its key downstream effectors Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) (75). When Hippo signaling is disrupted, YAP and TAZ translocate into the nucleus where they bind to the TEA Domain Transcription Factors (TEADs) promoting the expression of growth factors and apoptosis inhibitors (73, 76, 77). It has been reported that the development of primordial follicles is accompanied by an inhibition of the Hippo pathway (78, 79), while its overstimulation leads to a reduction in follicular proliferation and estrogen production in granulosa cells, both *in vivo*, and *in vitro* (80, 81). Before ovulation, oocyte-secreted factors contribute to the activation of YAP protein in granulosa cells stimulating their proliferation, whereas after ovulation, the Hippo pathway is transiently activated leading to YAP degradation, which allows the differentiation of granulosa cells into luteal cells and the production of progesterone (79).

There is an intrinsic mechanism that orchestrates the function of the mTOR and Hippo pathways through YAP and indirectly controls the granulosa cell–oocyte interactions. Interestingly, recent studies show that the communication of the Hippo pathway with the PI3K/Akt/mTOR axis and their coordinated regulation play a key role in follicular size and primordial maturity, through YAP and SMAD2/3 complex (48, 82, 83). Activation of the Akt/mTOR pathway using Akt stimulators in combination with inhibition of Hippo through ovarian fragmentation appears to increase the number of mature follicles in mouse models, but also in patients with POI or PCOS,

adjusting follicular growth and ovulation, thereby leading to successful fertilization and pregnancy (49, 52, 84, 85). Cytoskeleton remodeling is one of the key factors regulating Hippo signaling and promoting the nuclear localization of YAP/TAZ complex (86). Importantly, recent findings in mouse models show that hMG administration leads to activation of the mTOR pathway (87), and GnRH induces cytoskeleton reorganization (a key process for the synthesis and secretion of gonadotropins) by activation of the mTOR kinase (88). Actin cytoskeleton dynamics mediates vital roles, also, for oocyte meiotic cell divisions through Hippo and mTOR signaling (89–91). In early stage oocytes (germinal vesicle) YAP is predominantly located in the cytoplasm, whereas during the subsequent stages of oocyte development (metaphase I), YAP becomes activated and translocates into the nucleus, suggesting a role of Hippo signaling in oocyte maturation (92). In addition, the mTOR pathway plays fundamental role on oocyte meiotic maturation through the activation of translation of specific mRNAs involved in spindle morphology and chromosomal alignment (93, 94). Consistently, disruption of mTOR signaling inhibits spindle migration and asymmetric division in mouse oocytes (95).

Thus, it becomes evident from the above that a better understanding of the way that the different gonadotropin regimens affect the PI3K and Hippo pathways within the follicular environment in women with reduced ovarian reserve, polycystic ovary syndrome or advanced maternal age will allow their use as potential benchmarks for guidance of physicians regarding more efficient strategies for IVF (**Figure 1**).

CONCLUDING REMARKS

It is clear, that further randomized controlled studies are needed to investigate the effects of the different gonadotropin preparations in the IVF outcome, and importantly, to combine both clinical and molecular attributes in order to appreciate the ovarian biological underpinnings of the various treatments. A better knowledge of the effects of the various gonadotropin preparations on the activation of follicles will allow the elaboration of appropriate biomarkers which in turn will render it possible to evaluate the efficacy of the different stimulation protocols in *in vitro* fertilization in different groups of patients. Current evidence reveals the presence of an active

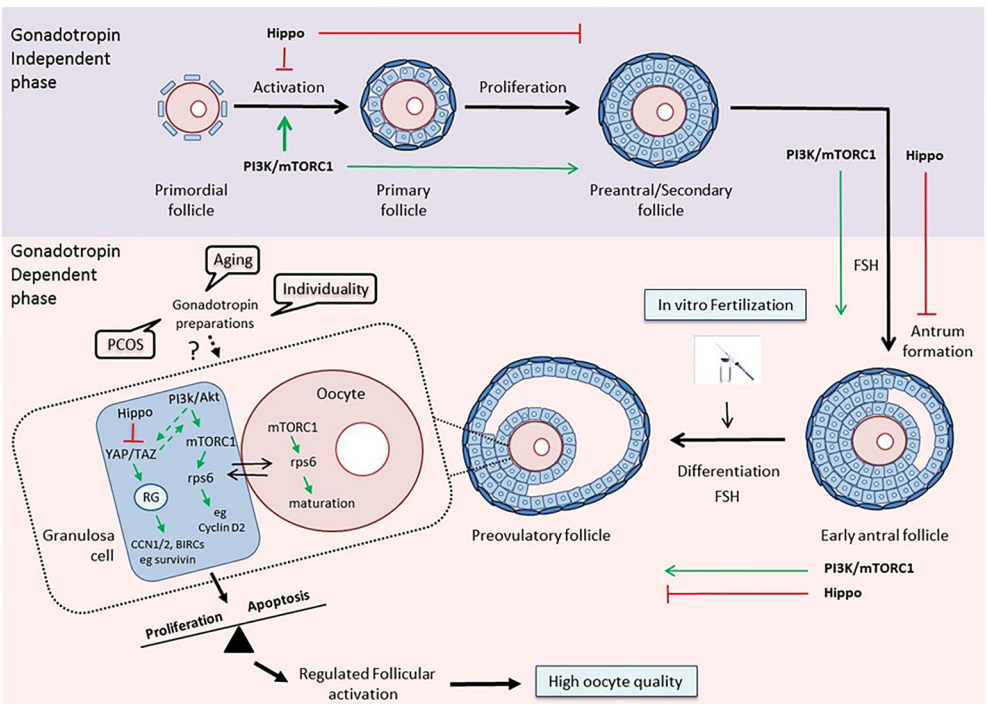

FIGURE 1 | The PI3K/mTOR/Hippo pathways as guidance for clinical decision-making. Top: The PI3K/Akt/mTOR and Hippo pathways exert opposite effects on follicular development during the gonadotropin-independent phase. Activation of the PI3K pathway is crucial for each growing stage of the follicle, especially at the primordial and primary stages (30, 94). The Hippo pathway acts in a coordinated manner with PI3K in order to accelerate primordial follicle activation and promote follicular development (48). Bottom: The two pathways maintain their concerted action on follicular development during the gonadotropin-dependent phase of follicular growth, and especially on the maturation of granulosa cells and oocytes in the preovulatory follicles, thereby assuring regulated follicular activation and high oocyte quality (79, 96). Various disease states, aging, and the uniqueness of each woman, by influencing this balance, may affect the response to different gonadotropin preparations, and consequently, the outcome of the IVF. The activation status of key components of the PI3K and Hippo pathways may serve as a prognostic or predictive biomarker that can help clinicians guide treatment planning. (RG, Regulatory Genes).

cross-talk between the PI3K/Akt/mTOR and the Hippo pathways, which is instrumentally involved in the regulated activation of primordial follicles, as well as, in follicular and oocyte growth. Consequently, a deeper understanding of the influence of the various ovarian stimulation protocols might exert on this interplay could help scientists to translate the emerging novel knowledge into clinical success and contribute to more efficient management of assisted reproduction methods. However, this is not an easy task. Despite the substantial progress in understanding ovarian follicular physiology, ART remains an inefficient process (97, 98). While the success rates of IVF/ART programs initially displayed an upward trend, the pregnancy and birth rates are declining in recent years (3). This issue has been thoroughly discussed by Norbert Gleicher and co-workers (99). Apparently, there are several causes, including potentially harmful add-ons to IVF practice, the woman's age that dramatically influences the responses to exogenous gonadotropin stimulation (100–102) but also an evolving industrialization and commoditization of IVF (99). Considering the heterogeneity of the infertile population, understanding the best gonadotropin regimen for a particular patient necessitates two prerequisites. On the one hand, a personalized tailored approach (103, 104) which implies that we need to understand the mechanisms by which the same protocol results in different outcomes in different women, for example by monitoring gene expression profiles (105–108). On the other hand, the international cooperation between fertility societies such as ESHRE (European Society of Human Reproduction and Embryology), ASRM (American Society for Reproductive Medicine), or IFFS (International Federation of Fertility Societies) as well as Delphi Consensus statements, which by continuing to periodically update progress in basic research and reinforcing the dissemination of evidence-based information can facilitate and foster the translation of basic research into clinical practice.

In the long term, the elaboration of more straightforward and simple testing procedures based on key signaling cascades governing granulosa cell biology will help clinicians to prevent their patients from unnecessary treatment, and hopefully, will lead to more effective and individualized treatment protocols to improve birth rates.

AUTHOR CONTRIBUTIONS

KP and TM contributed to text conception. TM wrote the manuscript and KP has generated the figure. All authors contributed to the article and approved the submitted version.

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Evaluation of Ovarian Reserve Tests and Age in the Prediction of Poor Ovarian Response to Controlled Ovarian Stimulation—A Real-World Data Analysis of 89,002 Patients

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Aims: This study aimed to explore the value of ovarian reserve tests (ORTs) for predicting poor ovary response (POR) and whether an age cutoff could improve this forecasting, so as to facilitate clinical decision-making for women undergoing *in vitro* fertilization (IVF).

Methods: A retrospective cohort study was conducted on poor ovary response (POR) patients using real-world data from five reproductive centers of university-affiliated hospitals or large academic hospitals in China. A total of 89,002 women with infertility undergoing their first traditional ovarian stimulation cycle for *in vitro* fertilization from January 2013 to December 2019 were included. The receiver operating characteristic (ROC) curve was performed to estimate the prediction value of POR by the following ORTs: anti-Mullerian hormone (AMH), antral follicle count (AFC), basal FSH (bFSH), as well as patient age.

Results: In this retrospective cohort, the frequency of POR in the first IVF cycle was 14.8%. Age, AFC, AMH, and bFSH were used as predicting factors for POR, of which AMH and AFC were the best indicators when using a single factor for prediction (AUC 0.862 and 0.842, respectively). The predictive values of the multivariate model included age and AMH (AUC 0.865), age and AFC (AUC 0.850), age and all three ORTs (AUC 0.873). Compared with using a single factor alone, the combinations of ORTs and female age can increase the predictive value of POR. Adding age to single AMH model improved the prediction accuracy compared with AMH alone (AUC 0.865 *vs.* 0.862), but the improvement was not significant. The AFC with age model significantly improved the prediction accuracy of the single AFC model (AUC 0.846 *vs.* 0.837). To reach 90%

specificity for POR prediction, the cutoff point for age was 38 years old with a sensitivity of 40.7%, 5 for AFC with a sensitivity of 55.9%, and 1.18 ng/ml for AMH with a sensitivity of 63.3%.

Conclusion: AFC and AMH demonstrated a high accuracy when using ROC regression to predict POR. When testing is reliable, AMH can be used alone to forecast POR. When AFC is used as a prediction parameter, age is suggested to be considered as well. Based on the results of the cutoff threshold analysis, AFC ≤ 5 and AMH ≤ 1.18 ng/ml should be recommended to predict POR more accurately in IVF/ICSI patients.

Keywords: poor ovary response, *in vitro* fertilization/intracytoplasmic sperm injection, female age, real-world study, ovarian reserve tests

INTRODUCTION

Predicting a patient's ovarian response prior to the start of the first IVF cycle is important in clinical practice for providing important diagnostic and prognostic value.

Poor ovary response (POR) is characterized by a low number of growing follicles and low serum estradiol levels after exogenous gonadotropin stimulation, resulting in a poor oocyte retrieval. POR is associated with poor reproductive outcomes (1, 2). According to the consensus elaborated by the European Society of Human Reproduction and Embryology (ESHRE) in 2011, to define POR, at least two of the following three features must be present: (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 (i.e., AFC of 5–7 follicles or AMH of 0.5–1.1 ng/ml). Two episodes of POR after maximal stimulation are sufficient to define a patient as a poor responder in the absence of advanced maternal age or abnormal ORT. From that time, according to the literatures, the prevalence of POR after ovarian stimulation ranged from 5.6 to 35.1% worldwide (3), and it relates to poor IVF outcomes and low pregnancy rate for these patients (4).

In the past few decades, numerous studies have been carried out to measure ovarian reserve through ovarian reserve tests (ORTs) (5–10). Basal FSH (bFSH) plus estradiol levels or AMH are recommended as most appropriate ovarian reserve screening tests. According to increasing numbers of studies, AMH and antral follicle count (AFC) represent direct and accurate measurements of the ovarian follicle pool (11, 12). ORTs are often used in combination to improve the prediction of POR. However, according to the past meta-analysis (6, 13), combinations of a few tests only show a minimal improvement in prediction of POR when compared with using a single test. The lack of improvement might be explained by the heterogeneity of the tests and the cutoff points used in different research studies. Furthermore, ORTs only define ovarian reservation quantitatively, while the best surrogate marker for oocyte quality is age (6, 14, 15). ORT results, combined with age, could be useful for discussing a patient's prognosis and recommending a treatment plan in practice. Thus, in order to forecast POR, using age in addition to ORTs should be investigated. More specifically, an optimal combination of measurements should be determined, which considers differences in methods of measuring hormone and the definition of uniform POR.

The study benefited from the establishment of a multicenter retrospective database and used a large sample of 89,002 patients who underwent their first *in vitro* fertilization (IVF) cycles in China to analyze the accuracy of POR prediction by female age and ORTs alone and in combination. The study also explored the cutoff points of key indicators to predict POR and stratified cutoff point according to age.

METHODS

Study Cohort and Data Acquisition

This study included the first oocyte retrieval IVF/ICSI cycle of all patients from January 2013 to December 2019 at five reproductive centers in university-affiliated hospitals or large academic hospitals in China including the Sixth Affiliated Hospital of Sun Yat-sen University, Henan Provincial People's Hospital, Jiangsu Provincial People's Hospital, Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology, and Northwest Women's and Children's Hospital. The study was reviewed and approved separately by the ethical committees in each hospital, namely, the Reproductive Medicine Ethics Committee of Henan Provincial People's Hospital (SYSZ-LL-2019110401), Medical Ethics Committee of Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology (TJ-IRB20210320), Medicine Ethics Committee of Jiangsu Provincial People's Hospital (2020-SR-046), Medical Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University (2020ZSLYEC-295), and Medical Ethics Committee of Northwest Women's and Children's Hospital (2019013). The need for individual consent was waived by the committees due to the retrospective character of the study. Data was desensitized to hide personal information before being processed.

The raw data came from the IVF database of the five reproductive centers. We retrieved the desensitization data of patients who underwent IVF/ICSI treatment from January 2013 to December 2019 from each center. The types of raw data

collected included hospital admission summary sheet, medical history records of the couples, cycle information, ovulation monitoring, oocyte retrieval records, embryo culture records, frozen and thawing records, transplant records, follow-up records. Data were processed from medical records into standardized research datasets for further analysis.

Inclusion and Exclusion Criteria

Inclusion criteria: Female patients with regular menstruation and bilateral ovaries at one of the five reproductive centers with first-time fresh cycles of IVF from January 2013 to December 2019 were included in the analysis.

Exclusion criteria: Patients with evidence of any of the following conditions were excluded from the study: ① polycystic ovarian syndrome (PCOS) (according to Rotterdam Criteria); ② history of ovarian surgery; ③ history of chemotherapy and pelvic radiotherapy; ④ pretreatment of oral contraceptives within 2 months before conducting the IVF cycle; ⑤ natural cycle IVF and mild stimulation cycle with daily gonadotropin (Gn) <150; ⑥ canceled oocyte retrieval cycle that isn't due to poor ovarian response.

Treatment

Every patient that met the inclusion criteria underwent the first *in vitro* fertilization cycle. The stimulation protocol and the dose of gonadotropin were determined by the reproductive endocrinologist. In all cases, the dose of gonadotropin was chosen to optimize the number of oocytes retrieved while minimizing the risk of ovarian hyperstimulation syndrome (OHSS).

Before the cycle, venous blood was collected on days 2–4 of the menstrual cycle, and the AFC was measured through a transvaginal ultrasound examination by a reproductive endocrinologist or an experienced sonographer. Within one center, these posts are filled by relatively permanent personnel. Since all the five reproductive centers are large artificial reproductive technology centers of China and each center has its own personnel training and assessment process, thus, the results of the AFC were reliable. AFC is defined as the number of 2–10 mm diameter follicles in two ovaries. After standard venipuncture, the blood sample was completely coagulated and the sample was centrifuged. Then 1 ml serum was removed to a new tube, frozen at 2–8°C within 24 h after blood collection, and tested in an independent laboratory of each IVF center within 2 days. Kangrun Biotech Reagent Automatic SMART6500 immunoassay analyzer was used to detect levels of AMH and sex hormones in serum and plasma samples. The published total imprecision of the AMH assay kit was 2.4–5.2% (16, 17).

Definition and Statistics

POR is defined as the cancelation of the oocyte retrieval cycle due to poor ovarian response or cycles in which the number of oocytes retrieved is three or fewer (5, 6, 18). ORTs include bFSH, AMH, and AFC.

POR was designated as the dependent variable (1=POR; 0=enough to achieve high ovarian response), and the following continuous variables were used as independent variables in the analysis: AMH, AFC, bFSH, age. Receiver operating characteristic (ROC) analysis was used to map the sensitivity and specificity of the four independent variables, in order to predict the POR of all possible cutoff points for each indicator. The area under the ROC curve and 95% CI were then described. According to previous studies (3, 18), the cutoff points are typically determined to be the value when the specificity of predicting POR is 90%. Maximizing specificity was the goal in this study in identifying a cutoff point for predicting POR to avoid overestimating the risk of POR (6, 11). The ideal screening test should demonstrate high specificity to minimize the risk of a false-positive determination of decreased ovarian reserve in a woman with normal ovarian reserve (11, 18). Therefore, the cutoff point that maximizes specificity is preferred, even if it means reduced sensitivity. Statistical tests were two-sided tests, and P-value <0.05 was considered statistically significant. All analyses used R language.

RESULTS

Baseline Patients and Cycle Characteristics

Five large- and medium-sized reproductive centers located in different regions of China (east, west, south, north, and middle area) conducted a total of 327,059 IVF/ICSI cycles, of which 145,158 (44.38%) were fresh cycles of first ovulation induction, and at last 89,002 cycles were eligible. Among them, 48,642 cases (54.65%) had AMH test results, 41,702 cases (46.86%) of which used the same detection method (electrochemiluminescence method, Kangrun Biotech); 85,052 cases (95.56%) had bFSH test results; 88,987 cases (99.98%) were recorded with age; 84,884 cases (58.47%) were recorded with AFC. The specific inclusion and exclusion process of patients is shown in **Figure 1**.

Table 1 describes the baseline characteristics of these patients, including demographic information and ORT parameters. All study subjects were Chinese women (N=89,001), with an average age of 32.0 ± 5.1 years and an average BMI of 22.4 ± 3.1. Women over 35 years old accounted for 23.9%, and women over 38 years old accounted for 12.1% of the study population. The most common indications of IVF treatment were pelvic tubal factors (47.1%), male factors (15%), and ovarian factors (14.6%). The average AMH level was 3.6 ± 3.0 ng/ml, the average AFC was 11.1 ± 5.5, and the average bFSH was 7.7 ± 3.3 mIU/ml. Commonly used ovulation induction protocols included GnRH agonist protocol (69.1%), GnRH antagonist protocol (22.2%), progestin-primed protocol (2.2%), and COS protocol without ovarian suppression (6.55%). The median total dose of Gn was 2,200.0 [1725.0,2775.0] IU (quartile), the average was 2,320.4 ± 922.0 IU; the median Gn use days was 10.0 [9.0,11.0] days (quartile), and the average is 10.1 ± 2.5 days. During the process of Gn usage, recombined FSH accounted for 54.01%, and HMG was added to the latter stage of COS in 97.6% of the cycles. The average daily Gn average was 228.5 ± 65.8 IU. POR occurred in 13,196 patients (14.8%).

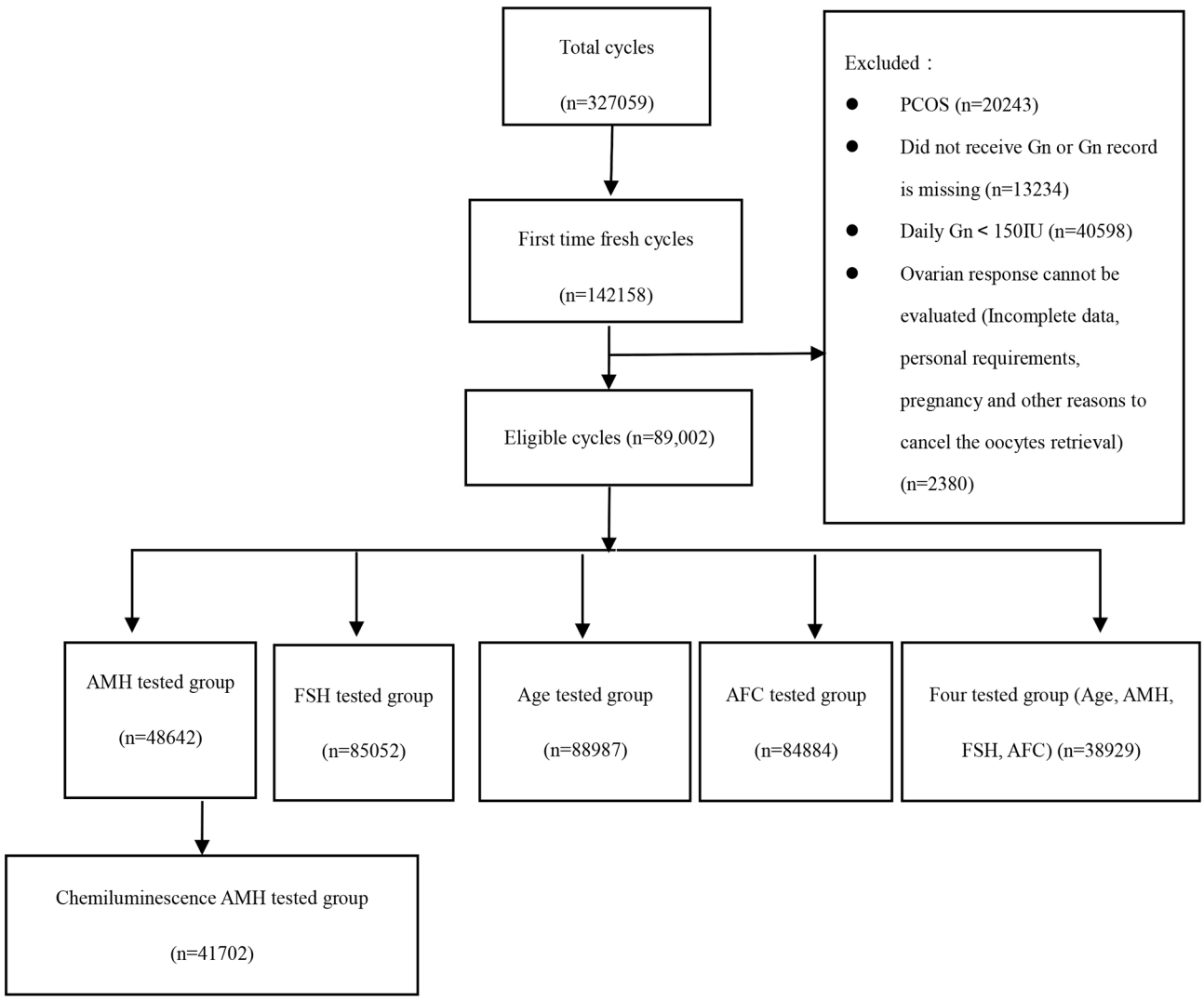

FIGURE 1 | The data processing.

Regression Analysis and ROC Curve

Multivariate logistic regression showed that age was significantly associated with POR with an odds ratio (OR) (95% CI) of 1.050 (1.040–1.059); AFC, AMH, and bFSH were also significantly associated with POR with OR (95% CI) of 0.898 (0.886–0.912), 0.712 (0.672–0.754), and 1.090 (1.073–1.106), respectively (P<0.001) (shown in affiliated table). Age, an independent influencing factor on pregnancy outcomes, was correlated with the other predictors in the study, so we created two models for the prediction of POR. The first models were univariate models for each of the ORTs (bFSH, AFC, or AMH) and age as predictors separately. The second models were multivariate models that evaluated combinations of each ORT and age together. The different models were used to parse out the predictive value of age and ORTs alone, as well as the added predictive value of age to AMH, AFC, bFSH in combination. ORT parameters combined with age significantly improved the prediction of POR after control ovarian stimulation (COS) (see in **Table 2**).

Based on the results from the models in **Table 2**, we constructed the ROC curve for each factor of the ORTs and the combination of age and each ORTs that predicted POR with statistical significance. Next, we compared the area under the curve. Due to the nature of this retrospective analysis, not all parameters of each patient were complete. Therefore, we drew the ROC curve on (i) the whole population (whole group) (N=89,001) and (ii) the patients with complete data (four tested groups) (N=38,929). As the detection of AMH was updated from the previous ELISA method to the current electrochemiluminescence method, we only studied patients whose AMHs were measured by electrochemiluminescence (41,702 cases) to exclude the influence of different detection methods.

The multivariate analysis of POR prediction showed that the prediction accuracy in the combined model with all predictors

TABLE 1 | Baseline characteristics and treatment outcome of the 89,001 women in the study group.

Participant characteristics	Mean ± SD
Female age*	32.0 ± 5.1
Female BMI*	22.4 ± 3.1
Infertility duration*	4.0 ± 10.3
Infertility factors*, No. (%)	
Ovary factor	11,963 (14.6)
Male factor	12,268 (15.0)
Pelvic or tubal factor	38,586 (47.1)
Genetic factor	3,209 (3.9)
Uterine or cervix factor	6,283 (7.7)
Endometriosis	6,014(7.3)
Other factors	3,570 (4.4)
Basal AMH*, Mean ± SD	3.6 ± 3.0
BasalE2*, Mean ± SD	47.6 ± 29.6
AFC*, Mean ± SD	11.1 ± 5.5
Basal bFSH*, Mean ± SD	7.7 ± 3.3
COS Protocols*, No. (%)	
GnRH agonist protocol	57,630 (69.1)
GnRH antagonist protocol	18,512 (22.2)
Progestin-primed protocol	1,875 (2.2)
No ovary suppression	5,437 (6.55)
POR, No. (%)	
No	75,805 (85.2)
Yes	13,196 (14.8)

**Data not available for all subjects. Missing values: female age = 14, female BMI = 647, infertility duration = 4,179, female age group = 14, female. BMI group = 647, infertility type= 1,711, infertility factor = 7,108, protocol group = 5,547, AFC = 4,117.*

AFC, AMH, bFSH, and age was higher than that of the models based on only one parameter. The AUC (95% CI) of the combined model was 0.873 (0.868–0.879). The AUC of the combined model was significantly better than the predicted value of a single parameter, but not significantly better than AMH plus age with AUC (95% CI) of 0.865 (0.860–0.870). The model with AMH alone had the highest AUC (AUC 0.862) among the univariate prediction models; followed by AFC (AUC 0.842). Adding age to the AMH model did not significantly improve the prediction accuracy (AUC 0.865 with age *vs.* AUC 0.862 without age). On the other hand, the age plus AFC model significantly improved the prediction accuracy of the single AFC model (AUC 0.846 with age *vs.* AUC 0.837 without age). The AUC of bFSH was relatively small in comparison to the other predictors, AUC 0.689 (0.683–0.695), and the predictive effects of single and combined bFSH use were both moderate. Details can be seen in **Table 3**.

ROC curves of univariate and multivariate models are shown in **Figure 2**.

Cutoff Points

For each predictor, the cutoff point was determined based on specificity of about 90% for predicting POR. We report the values for the predictors at 90% specificity and their sensitivities. The ROC analysis found that to predict POR, a cutoff point of 38 years old yielded a sensitivity of 40.7%; the cutoff point of bFSH was 9.8 mIU/ml with a sensitivity of 38.4%; the cutoff point of AFC was 5 with a sensitivity of 55.9%; the cutoff point of AMH was 1.18 ng/ml with a sensitivity of 63.3%. Comparing these factors used independently for POR prediction, AMH achieved the highest sensitivity with 90% specificity. AMH levels below 1.18 ng/ml were associated with a higher incidence of POR. After stratifying by age group, for patients younger than 35 years old, the cutoff point for AMH is 1.37 ng/ml and for AFC is 6. Details can be seen in **Table 4**.

DISCUSSION

These results of this real-world study, based on a multicenter retrospective study of 89,002 patients, indicate that age, AFC, AMH, and bFSH are predicting factors for POR, of which AMH and AFC are the best indicators when using a single factor for prediction. Age improves the above predictions with a cutoff point of 38 years old. When testing is reliable, AMH can be used alone to forecast POR. However, while AFC is used as a prediction parameter, we suggest that female age should be included at the same time for reference.

In the long-lasting debate on the true value of ORTs prior to IVF, a real-world study can be of help as an objective approach in

TABLE 2 | Univariate and multivariate models of age and ORT in the prediction of POR.

	N	OR (95% CI)	p-value
Univariate models			
Age (per year)	88,987	1.183 (1.179–1.188)	<.0001
bFSH (per IU/L)	85,052	1.258 (1.250–1.266)	<.0001
AFC (per N)	84,884	0.707 (0.702–0.711)	<.0001
AMH (per ng/ml)	41,702	0.370 (0.359–0.382)	<.0001
Multivariate models			
Age and bFSH			
Age (per year)	85,041	1.164 (1.159–1.169)	<.0001
bFSH (per IU/L)	85,041	1.219 (1.211–1.227)	<.0001
Age and AFC			
AFC (per N)	84,872	0.736 (0.731–0.741)	<.0001
Age (per year)	84,872	1.086 (1.081–1.091)	<.0001
Age and AMH			
Age (per year)	41,695	1.084 (1.077–1.090)	<.0001
AMH (per ng/ml)	41,695	0.412 (0.400–0.425)	<.0001

TABLE 3 | AUCs of prediction models of age and ORTs for the prediction of POR.

	Total group		Four-tested group	
ROC Model	**AUC (95% CI)**	**n**	**AUC (95% CI)**	**n**
Univariate models				
Age	0.723 (0.718–0.728)	88,987	0.712 (0.704–0.720)	38,929
bFSH	0.689 (0.683–0.695)	85,052	0.681 (0.673–0.690)	38,929
AFC	0.842 (0.838–0.846)	84,884	0.837 (0.832–0.843)	38,929
AMH	0.862 (0.857–0.867)	41,702	0.858 (0.852–0.864)	38,929
Multivariable models				
Age+bFSH	0.773 (0.769–0.778)	85,041	0.765 (0.757–0.772)	38,929
Age+AFC	0.850 (0.846–0.854)	84,872	0.845 (0.839–0.850)	38,929
Age+AMH	0.865 (0.860–0.870)	41,695	0.862 (0.856–0.867)	38,929
Age+bFSH+AFC+AMH	0.873 (0.868–0.879)	38,929	0.873 (0.868–0.879)	38,929

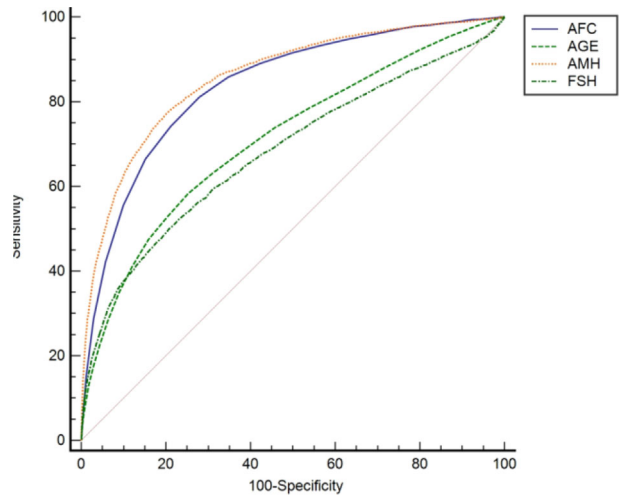

FIGURE 2 | ROC curves of the POR prediction model by each parameter.

summarizing the available evidence. The real-world study results are more representative of usual clinical practice and have important guiding significance for clinical practice (19). With the cooperation of various centers, we were able to collect a great number of cases with good homogeneity and were able to explore prediction of POR in Chinese infertile populations. In addition, this study adds to a body of literature describing predictors of POR that have historically been defined according to the Bologna criteria and the Poseidon criteria. Furthermore, we screened instruments, methods, and reagents of AMH measurement to accommodate the heterogeneity between centers.

The findings from our analysis confirm those of previous systematic reviews and meta-analysis of both single ORTs and multivariable prediction models for POR to control ovarian stimulation (6, 20, 21). Both AMH and AFC strongly represent the size of the cohort of FSH-sensitive follicles in the ovaries, thus often referred to as the quantitative ovarian reserve. AFC and AMH are highly correlated and also have discordance (22, 23). Comparing AMH with AFC, AMH has the advantage of very little intra- and inter-cycle variability (24). When challenged against AFC, AMH level is not only a quantitative but also a

TABLE 4 | Cutoff point analysis—total group and age stratification.

Variable	Cutoff point	Sensitivity	Specificity	Youden index
Age				
Total	≤38	0.407	0.890	0.296
bFSH				
Total	≤9.8	0.384	0.900	0.283
<35	≤9.62	0.354	0.900	0.254
35–38	≤10.18	0.351	0.900	0.251
38–40	≤10.49	0.362	0.900	0.262
>40	≤11.51	0.320	0.900	0.220
AFC				
Total	≤5	0.559	0.908	0.467
<35	≤6	0.538	0.895	0.434
35–38	≤4	0.377	0.925	0.303
38–40	≤3	0.319	0.933	0.252
>40	≤3	0.465	0.875	0.340
AMH				
Total	≤1.18	0.633	0.900	0.534
<35	≤1.37	0.607	0.900	0.508
35–38	≤1.02	0.538	0.901	0.438
38–40	≤0.8	0.493	0.899	0.392
>40	≤0.61	0.518	0.899	0.417

qualitative follicle marker, in relation with clinical and endocrine parameters (25, 26). Through our study, we conclude that AMH is the best independent predictor of POR, when comparing other ORTs and age separately as predictors. Historically, there were issues with AMH's low comparability of measured values between clinical laboratories; however, recent advances in new automated assays have greatly improved repeatability and comparability (27). For all the cases included in our model, AMH is tested by Access AMH with electrochemiluminescence detection, which is more accurate than the ELISA method (28, 29). The predictive effect of AFC has often been questioned because of variability in the operator's technical proficiency. However, our study shows that across various centers, AFC was a good predictor of POR. This may be related to the fact that all the centers participating in the study are large reproductive centers in China, with well-trained sonographers, advanced ultrasound equipment, and standardized management. Nevertheless, age improves the prediction of AFC significantly.

The clinical use of markers like AMH, bFSH, and AFC is mostly based on cutoff points. From the individual patient dataset, cutoff points for poor response prediction could be derived that have general applicability. Unfortunately, cutoff points reported in literature are very variable (3, 11, 18). Such variability could be explained by factors such as the low number of subjects included in some of these studies, the variability in the measuring methods used for these markers, and the different definitions of POR. According to published studies, cutoff points of AMH range 0.10–1.66 ng/ml, with reported sensitivities of 44–97% and specificities of 41–100%; cutoff points of AFC range between 3–10, with reported sensitivities of 9–73%; and specificities of 73–100% (13, 17). Our study shows that for predicting POR, the cutoff points of AMH and AFC were 1.18 ng/ml and 5, respectively, for predicting POR in the whole population, ranging between 0.61–1.37 and 3–6 in different age groups, and decreased with age. For younger women (less than 35 years old and 35~38 years old), cutoff points of AMH and AFC were 1.37 and 1.02 ng/ml, 6 and 4. These results may help recognize and intervene in young patients with ovarian reserve decline. Recent publications have also suggested the calculation of age-specific ovarian reserve decline curves in order to maximize ORT accuracy (30, 31).

The cutoff point of age is 38 years (specificity 89%, sensitivity 40.7%, AUC 0.723), which differs from the existing 40 years old cutoff point in the Bologna criteria and 35 years old cutoff point in the Poseidon categories. It was also reported with given evidence from multiple studies that the average rate of follicular depletion, aneuploidy rate, and embryo arrest rate all increase significantly after age 38 (32–34).

A limitation of this study is that although Access AMH with electrochemiluminescence detection was used, variability between different laboratories in each center is still worth exploring. Also, pregnancy outcomes and effective management strategies of POR patients are not referred to. These should be explored on the basis of this research in the future.

In conclusion, POR is estimated to occur in 14.8% of the first IVF cycles in the Chinese population. When testing is reliable, AMH can be used alone to forecast POR. When AFC is used as a prediction parameter, age is suggested to be considered as well. AFC ≤5, AMH ≤1.18 ng/ml, and female age ≥38 should be recommended to predict POR more accurately in IVF/ICSI patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Henan Provincial People's Hospital, Ethics Committee of Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology, Ethics Committee of Jiangsu Provincial Hospital, Ethics Committee of Northwest Women's and Children's Hospital, and Ethics Committee of Sixth Affiliated Hospital of Sun Yat-sen University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

C-LZ and X-YL supervised the entire study, including the procedures, conception, design, and completion, and participated in the interpretation of the study data and in revisions to the article. LJ, J-ZS, Y-DM, and RH were responsible for the collection of data. XW contributed to the data analysis and drafted the article. Y-NJ wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.702061/full#supplementary-material

Supplementary Table 1 | Univariable and multivariable models of age and ORTs in the prediction of poor response.

Supplementary Table 2 | Univariable and multivariable models of age and ORT in the prediction of poor response.

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The Exploration of Poor Ovarian Response–Related Risk Factors: A Potential Role of Growth Differentiation Factor 8 in Predicting Ovarian Response in IVF-ET Patient

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Controlled ovarian hyperstimulation (COH) is the most common therapeutic protocol to obtain a considerable number of oocytes in IVF-ET cycles. To date, the risk factors affecting COH outcomes remain elusive. Growth differentiation factor 8 (GDF-8), a member of transforming growth factor β (TGF-β) superfamily, has been long discerned as a crucial growth factor in folliculogenesis, and the aberrant expression of GDF-8 is closely correlated with the reproductive diseases. However, less is known about the level of GDF-8 in IVF-ET patients with different ovarian response. In the present study, the potential risk factors correlated with ovarian response were explored using logistic regression analysis methods. Meanwhile, the expression changes of GDF-8 and its responsible cellular receptors in various ovarian response patients were determined. Our results showed that several factors were intensely related to poor ovarian response (POR), including aging, obesity, endometriosis, surgery history, and IVF treatment, while irregular menstrual cycles and PCOS contribute to hyperovarian response (HOR). Furthermore, POR patients exhibited a decrease in numbers of MII oocytes and available embryos, thereby manifesting a lower clinical pregnancy rate. The levels of GDF-8, ALK5, and ACVR2B in POR patients were higher compared with those in control groups, whereas the expression level of ACVR2A decreased in poor ovarian response patients. In addition, clinical correlation analysis results showed that the concentration of GDF-8 was negatively correlated with LH and estradiol concentration and antral follicle count. Collectively, our observations provide a novel insight of ovarian response–associated risk factors, highlighting the potential role of GDF-8 levels in ovarian response during COH process.

Keywords: receptor, ART, risk factor, poor ovarian response, GDF-8

INTRODUCTION

Infertility is a global issue, and its incidence of fertile population shows a rampantly increasing trend nowadays. Since the advent of *in vitro* fertilization embryo transfer (IVF-ET) in 1978, IVF-ET technology has been adopted worldwide as an approach to overcome infertility problem in couples seeking medical assistance. With IVF technology undergoing several developmental stages, controlled ovarian hyperstimulation (COH) has gradually become a main therapeutic approach to promote ovulation and subsequently obtain an optimum number of mature oocytes. Clinically, the effect of COH relies on the ovarian response, which is divided into poor, normal, and hyper response (1, 2). The poor ovarian response (POR) likely leads to few retrieved oocytes and diminished clinical pregnancy rate (3), whereas the pathogenesis of which has not yet been clearly clarified. Owing to the absence of reliable markers for direct prediction, it is difficult for clinicians to assess the ovarian response of women undergoing COH. Instead, ovarian reserve, referred to as reflection of fertility performance, is utilized as an indirect marker to clinically evaluate the ovarian response of patients during the process of assisted reproductive technology (ART) (4). Hyper ovarian response (HOR) is another pathologic condition in COH. In the last few years, it is suggested by clinical investigations that patients with HOR have an increasing tendency to develop ovarian hyperstimulation syndrome (OHSS) (5). Thus, the precise prediction of HOR would be beneficial to preventing the occurrence of OHSS in ART. To date, multiple factors have been reported to act in parallel regarding ovarian response, in terms of age, body mass index (BMI), genetics, environment, etc. Nevertheless, it still seems challenging to precisely assess ovarian response due to the lack of valid markers. Therefore, exploration of ovarian response–related risk factors would be advantageous to the evaluation of ovarian function and ideal IVF outcomes.

The maintenance of a well-balanced follicular environment is essential for the follicle development. Cell–cell interaction, especially the coordinate crosstalk between the oocyte and its surrounding granulosa cells, plays a vital role in creating intrafollicular microenvironment suitably equipped for further maturation. Past experiments have shed the light on a variety of growth factors as active participants in the regulation of gamete-somatic cell communications. Growth differentiation factor 8 (GDF-8), belonging to transforming growth factor β (TGF-β) superfamily, has been indicated to be profoundly involved in the modulation of folliculogenesis (6). Results of immunohistochemistry analysis illustrated that GDF-8 is widely expressed in different size of human growing follicles (7). In addition, further clinical studies reveal the dynamic changes of serum GDF-8 during the COH process, suggesting a changing role of GDF-8 in the progressively different stages of folliculogenesis (8). Within the growth phase of follicle development, GDF-8 mediates the response of granulosa cell to gonadotrophins followed by taking an active part in ovarian steroidogenesis (9). Meanwhile, *in vitro* experiment demonstrates that GDF-8 inhibits the proliferation of human granulosa cells (10). Considered as a substantial modulator in ECM remodeling and cumulus-oocytes complex (COC) expansion, GDF-8 has been revealed as a potential element in the pathogenesis of polycystic ovary syndrome (PCOS) *via* clinical research (7, 8, 11, 12). Notably, two of the latest studies found that the concentrations of GDF-8 in follicular fluid is negatively correlated with IVF and pregnancy outcomes, which illuminate the possibility of GDF-8 to assess the ovarian function (8, 11). Accordingly, we hypothesize that GDF-8 tends to be a potential predictor to evaluate the ovarian response in COH process. In the present study, we explored the ovarian response–related risk factors and determined the expression of GDF-8 and its responsible receptors expression in different ovarian response patients during COH.

MATERIALS AND METHODS

Ethics Statement and Human Subjects

The study was approved by the Ethical Committee of the Women's Hospital, Zhejiang University School of Medicine, China (File No. 20180141). All participants signed a document of informed consent before participation of the study. All the subjects were obtained from 767 women (166 POR, 409 normal response, and 192 HOR) undergoing IVF-ET treatment in the Reproductive Center of Women's Hospital, School of Medicine, Zhejiang University from 2014 to 2015. The causes of infertility included fallopian tube factors, endometriosis, polycystic ovary syndrome, male factors, unexplained infertility, etc.

Controlled Ovarian Hyperstimulation Protocol

Based on patient's age, AFC, and basal endocrine condition, GnRHa long protocol or short protocol was selected. The long protocol was to start the intradermal injection of GnRHa in the middle of the luteal phase, which was the 21st day of menstrual cycle or the 7th day after the basal body temperature rose. On the 3rd day of the treatment cycle, daily intramuscular injection of rFSH (Gonal-F, Serono, Switzerland) was carried out, and Follicle monitoring under B ultrasound was started on the 8th day. When the average diameter of the largest follicles reached 18 mm or the average diameter of three follicles reached 16 mm, treatment of rFSH was halted and instead injection of 10,000 IU human chorionic gonadotropin (HCG, Organon, USA) was implemented. After 34–36 h, oocytes were collected by vaginal ultrasound-guided puncture. In short protocol, GnRHa was given from the second day of menstrual cycle, and rFSH was added from the third day of the treatment cycle. Recombinant follicle-stimulating hormone (FSH) was applied as gonadotropin, and the initial dose was determined according to the patient's age, the number of antral follicles, and the previous response to

gonadotropin. The following dose was adjusted based on follicle development and E2 monitoring. When the average diameter of the largest follicle reached 18 mm or the average diameter of three follicles reached 16 mm, the following treatment was performed as in long protocol described above.

Diagnosis of Ovarian Response

Poor Ovarian Response

(1) Advanced maternal age (≥40 years old) or any other POR risk factor; (2) A previous POR with oocytes obtained by conventional stimulation ≤3; (3) Abnormal ovarian reserve test (ORT) result in terms of antimüllerian hormone (AMH) <0.5–1.1 ug/L or antral follicle count (AFC) <5–7. Two of these three criteria are required for a POR diagnosis. In addition, 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 POR.

Hyper Ovarian Response

Serum estradiol (E2) >11,010 pmol/L (3,000 pg/m1) on human chorionic gonadotrophin (hCG) given day or number of eggs obtained >15 or 20 when the Gn dosage is ≤225 U/d.

IVF-ET

The obtained oocytes were fertilized *in vitro* according to conventional methods. After 18 h, the pronucleus and polar bodies were observed to evaluate the fertilization. Observation of embryo division was done on the second and third days after egg retrieval. Blastomere with regular morphology and less than 15% of fragment was regarded as a valid embryo. In the 4–5 weeks after embryo transfer, clinical pregnancy was diagnosed if the gestational sac was detected by vaginal ultrasound, or the ectopic pregnancy was confirmed by surgical pathology. Patients were diagnosed as biochemical pregnancy when HCG elevated but no embryo sac was observed. The canceled cycle was defined as the absence of transplantable embryos or the cancelation of the fresh embryo transfer due to OHSS.

Enzyme-Linked Immunosorbent Assay Measurement

The GDF-8 Quantikine ELISA Kits (DGDF80) were obtained from R&D System (MN, USA). The serum or follicular fluid samples were collected at the day of oocyte retrieval and storage at −80°C if not detected immediately. The concentrations of GDF-8 in serum or follicular fluid were determined by enzyme-linked immunosorbent assay (ELISA) analysis according to the manufacturer's instructions. The sensitivity of GDF-8 Quantikine ELISA Kit was 5.32 pg/ml. The intra- and inter-assay errors of the GDF-8 ELISA were 5.4 and 6, respectively.

Western Blot

After oocytes were retrieved, the human granulosa-lutein (hGL) cells were purified from follicular fluid mixture by using density centrifugation from follicular aspirates. The cells were lysed, and protein concentration of sample was examined. Total 20 ug protein were loaded and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After that, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) and blocked by Tris-buffered saline (TBS) containing 5% non-fat dry milk for 1 h at room temperature and incubated overnight at 4°C with GDF-8 (ab203076, abcam, 1:1,000) or α-Tubulin (sc-23948, Santa Crzu, 1:5,000) antibodies. The next day, the membranes were washed with TBS three times and then incubated in the appropriate HRP-conjugated secondary antibody for 30 min. Similarly, the membranes were washed with TBS for three times after secondary antibody incubation. Finally, the immunoreactive bands were detected with an enhanced chemiluminescent substrate (Bio-Rad). The intensities of the bands were quantified with Image-Pro Plus software (v4.5; Media Cybernetics, USA).

Statistical Analysis

All statistical analyses were conducted on SPSS17.0 software. Data were analyzed by bilateral t test and statistical significance was defined as $P < 0.05$. The Kolmogorov-Smimov Test and the Levene's Test were first performed on the two groups of patients. For data with normal distribution and homogeneous variance, independent sample T test was used. Whitney U Test was used for data with non-normal distribution and uneven variance. The chi-square test was used to analyze the correlation of the classified data, and the confounding and influencing factors were analyzed hierarchically. Odds radio (OR) >1 indicates risk factors. Finally, logistic regression model was constructed in variables with $P < 0.05$.

RESULTS

Comparison of Ovarian Response–Associated Risk Factors

A total of 767 women were included in the study, of which 166 showed POR, 409 showed normal ovarian response, and 192 showed HOR. Hierarchical analysis was used for the factors that may cause poor or HOR, and the calculated OR value was listed in detail (**Supplementary Tables 1**, **2**). Since the number of menstrual days and the years of infertility did not conform to the null hypothesis of normal distribution in the poor, hyper, or normal group, the independent sample Mann-Whitney U test and independent sample median test method were used, and the results showed no statistically significant difference ($P>0.1$) (**Supplementary Tables 1**, **2**). Therefore, it can be considered that the number of menstrual days and years of infertility were not the key factors affecting ovarian reactivity. Our results pointed that age, IVF cycles, BMI, endometriosis, surgical history, and abortion were highly correlated with poor response group compared with these in normal response group (**Table 1**). Multifactor and non-conditional logistic regression analyses were carried out for the factors influencing ovarian response, during which the least significant variables were eliminated gradually, with age ≥35 years old, endometriosis of stage III and IV, abortion ≥4 times

TABLE 1 | Logistic regression analysis results of statistically significant variables.

Grouping	Variables	B value	S.E.	Odds ratio (OR) (95% confidence interval)	*P* value
Poor response (n=166)	Age ≥ 35 years old	1.313	0.2	3.717 (2.509, 5.505)	0.000
	Endometriosis Stage III	1.562	0.678	4.768 (1.261, 18.021)	0.021
	Endometriosis Stage IV	1.643	0.49	5.173 (1.981, 13.507)	0.001
	Abortion times ≥4	1.218	0.625	3.383 (0.994, 11.503)	0.051
Hyper response (n=192)	Age ≥ 35 years old	–0.586	0.247	0.556 (0.343, 0.904)	0.018
	Underweight	0.695	0.35	2.003 (1.009, 3.977)	0.047
	PCOS	0.773	0.284	2.166 (1.241, 3.781)	0.007

finally entering regression equation. Coefficients of the four were positive and significant, indicating there was an association between the four potential risk factors and poor response, as shown in **Table 1**. Similarly, multifactor and non-conditional logistic regression analyses were also carried out for HOR-associated factors, and the results showed that PCOS, underweight, protective factors, and ≥35 years old were correlated with high response (**Table 1**). Age, irregular menstrual cycles, and PCOS were highly correlated with hyper-response group. Intriguingly, age was considered as the risk factor in both poor and hyper-response groups. However, age was positively correlated with poor response group, whereas negatively correlated with hyper-response groups.

Relationship Between Different Ovarian Response in IVF-ET and Clinical Outcomes

A retrospective analysis was performed on 200 cases of poor, 200 cases of normal, and 200 cases of high ovarian response patients receiving routine IVF-ET treatment (excluding ICSI treatment cycle) in reproductive medical center of our hospital during 2014–2015 due to tubal factors. The characteristics of patients are shown in **Table 2**. In different response group, the populations were subgrouped according to the age more or less than 35 years old. In the age more than 35 years old groups, we found that the reutilization rate was lower in the hyper-response group, whereas there was no difference in the poor response group compared with these in the normal group (**Table 2**). Meanwhile, the number of valid embryos was higher in the hyper-response group but lower in the poor-response group compared to these in the normal group (**Table 2**). In the group of age less than 35 years old, we demonstrated that there was no difference in hyper- and poor-response groups. However, the number of valid embryos was higher in the hyper-response group but lower in the poor-response group when compared to the normal group (**Table 2**). Clinical outcome analysis results showed that POR with age <35 years old showed significant decline in the numbers of MII oocytes, valid embryos, and clinical pregnancy rate compared to the normal-response group. While numbers of MII oocytes, fertilization rate, valid embryos, and cancelation rate of IVF cycle were higher in the hyper-response group than in the normal group and transferred embryos was less, no significant difference was detected in clinical pregnancy rate between the two (**Table 3**). When it comes to ≥35 years old group, number of MII oocytes, valid embryos, transferred embryos, and clinical pregnancy rate were obviously lower in the poor-response group than in the normal group. The number of MII oocytes and valid embryos in the hyper-response group was significantly increased compared with those in the normal group, and the number of transferred embryos and clinical pregnancy rate were decreased (**Table 3**).

The Concentrations of GDF-8 in Different Ovarian Response Patients

Numerous growth factors play essential roles in folliculogenesis *via* autocrine/paracrine manners. The levels of various growth factor are dynamically changing at different follicle development phase. The aberrant expression of growth factors would disrupt the balance of follicular microenvironment and subsequently impair the ovarian response. Accordingly, the changes of follicular growth factor level might be possible indicators of ovarian response during COH process. Exploring the potential ovarian response–related biomarkers will be indispensable for achieving a precise estimation of ovarian response. To evaluate the changes of GDF-8 level in different ovarian response patients, both serum and follicular fluid GDF-8 concentrations were determined by ELISA. Our results showed that both serum and follicular fluid levels of GDF-8 in POR groups were significantly higher than those in the normal-response groups (**Figures 1A, B**).

TABLE 2 | Laboratory results of different ovarian response groups.

Age	Group	Number of MII oocytes	Fertilization rate	Valid embryos number
< 35 years old	Poor response (n=166)	2.45 ± 1.28*	0.640 ± 0.27	1.74 ± 1.01*
	Normal response (n=409)	6.05 ± 3.29	0.598 ± 0.24	3.396 ± 2.17
	Hyper response (n=192)	10.72 ± 5.40*	0.539 ± 0.23*	5.99 ± 4.35*
≥ 35 years old	Poor response (n=166)	2.17 ± 1.25*	0.626 ± 0.29	1.74 ± 1.02*
	Normal response (n=409)	6.25 ± 2.48	0.630 ± 0.19	3.61 ± 1.82
	Hyper response (n=192)	10.13 ± 5.22*	0.563 ± 0.24	5.50 ± 3.55*

**Means compared with the normal-response group, significant difference between the two groups exists.*

TABLE 3 | Clinical outcomes of different ovarian response groups.

Age	Group	Number of transplanted embryos	Number of cycle canceled and its rate (%)	Number of biochemical pregnancy and its rate (%)	Number of clinical pregnancy and its rate (%)	Number of abortion and its rate (%)	Number of live birth and its rate (%)
<35 years old	Poor response (n=166)	1.50 ± 0.836	16 (15.39%)	1 (1.14%)	32 (40.32%)*	3 (7.69%)	33 (37.5%)
	Normal response (n=409)	1.68 ± 0.858	28 (17.07%)	1 (0.74%)	66 (48.53%)	9 (13.64%)	52 (38.24%)
	Hyper response (n=192)	1.001 ± 0.983*	73 (42.94%)*	3 (3.09%)	52 (53.61%)	11 (21.15%)	40 (41.24%)
≥35 years old	Poor response (n=166)	1.54 ± 1.03*	18 (18.75%)	3 (3.85%)	20 (25.64%)*	7 (35%)	10 (29.23%)
	Normal response (n=409)	2.17 ± 1.08	5 (13.89%)	2 (6.45%)	18 (58.065%)	5 (27.78%)	12 (38.71%)
	Hyper response (n=192)	1.67 ± 1.08*	4 (13.33%)	1 (3.85%)	7 (26.92%)*	1 (14.29%)	5 (19.23%)*

**Means compared with the normal-response group, significant difference between the two groups exists.*

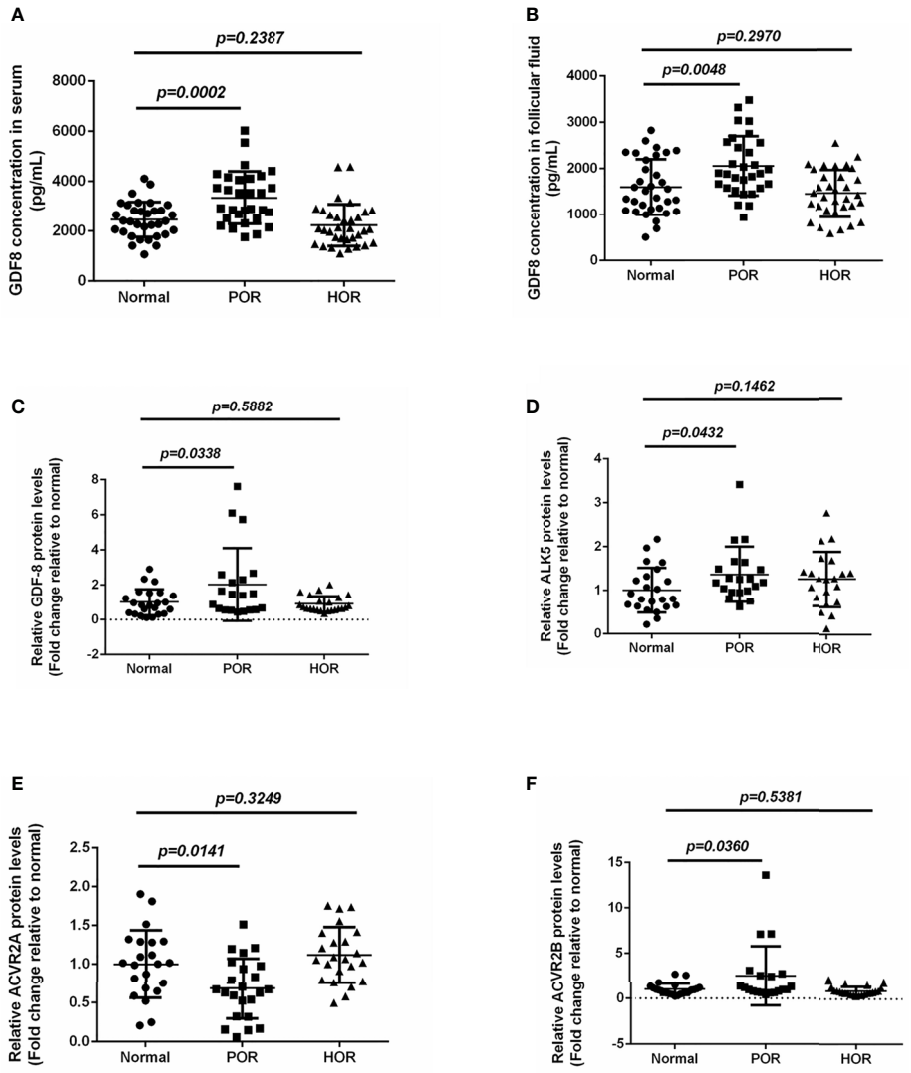

FIGURE 1 | The expression changes of GDF-8 and its responsible receptors in different ovarian response patients. **(A, B)** The accumulation level changes of GDF-8 in serum and follicular fluid obtained from different ovarian response patients were determined by ELISA. **(C–F)** The protein expression level changes of GDF-8 and its responsible receptors (ALK5, ACVR2A, and ACVR2B) were examined in different ovarian response patient granulosa cells using western blot. The data were analyzed by the two-sample t test assuming unequal variances. $P < 0.05$ was considered statistically significant. POR (n=30), poor ovarian response. HOR (n=33), hyper ovarian response. Normal, n=33.

However, there were no significant differences between normal- and hyper-response groups (**Figures 1A, B**).

The Expression Levels of GDF-8 in Human Granulosa Cells From Different Ovarian Response Patients

The activation of GDF-8-mediated signaling pathway is reliant on the combination of GDF-8 and its putative cellular receptors. It has been reported that GDF-8 activates the downstream signaling pathway by binding to ALK5, ACVR2A, and ACVR2B (7). Our previous study has demonstrated that GDF-8 and its putative cellular receptors ALK5, ACVR2A, and ACVR2B are distributed in intra cells of the follicle (7). To compare the difference of GDF-8 and its cellular receptor expression in human granulosa cells between different ovarian response patients, the protein levels of GDF-8 and its cellular receptors were examined by western blot. Our results found that GDF-8, ALK5, and ACVR2B protein levels in POR patients were higher than those in normal-response patients, whereas there was no difference between the hyper-response and normal-response groups (**Figures 1C, D, F**). Intriguingly, compared with normal-response patients, ACVR2A protein levels in POR patients were lower, and no difference was observed between hyper-response and normal-response patients (**Figure 1E**).

The GDF-8 Levels Are Negatively Correlated With Ovarian Response

To explore the correlation of GDF-8 with ovarian response, clinical information of patients undergoing IVF were collected. The correlation analysis results showed that GDF-8 levels were negatively correlated with LH and estradiol levels and antral follicle count (**Figures 2B–D**). However, there were no correlation between GDF-8 and FSH, AMH concentration (**Figures 2A, E**).

DISCUSSION

The female primordial follicle pool, established at birth, contains one million primordial follicles, thereby constituting primary ovarian reserve. However, only approximately 40,000 follicles exist when menarche occurs in adolescent as a result of atresia and degradation of most follicles during childhood. In each menstrual cycle, a number of primordial follicles are recruited and develop into growing follicles, but only one dominant follicle could be mature and then ovulated. Unfortunately, the number of primordial follicles would experience a double decline after the age of 40 and ultimately eliminated at menopause. Thus, the ovarian reserve of women is gradually reducing with enhancement of age. In our present study, we also demonstrated that ovarian response was negatively correlated with women age, in which ovarian response became worse with the increasing age. The negative correlation of both ovarian response and ovarian reserve with aging underpins the view that ovarian reserve is able to partially predict the ovarian response (4). Meanwhile, our results also revealed that young women were more likely to suffer from hyper response. Besides, previous studies have elucidated that overweight is related to decreased ovarian response, implying obesity as a risk factor affecting ovarian response (13). Our data also confirmed that obesity (BMI ≥25 kg/m^2) was a high risk factor of POR. Interestingly, it has been reported that high BMI does not affect the pregnancy outcomes of IVF in women, derived from the fact that the quality of oocyte obtained by COH has no significant difference between normal and high BMI women (14).

Polycystic ovary syndrome (PCOS) is a common gynecological endocrine disease in women of childbearing age, with an incidence of 5–10% worldwide. Meanwhile, PCOS is a complicated disease affecting multiple systems including the reproductive system. PCOS patients have abnormal follicular development, which is mainly manifested as excessive numbers of follicles in the early growth stage. PCOS patients have more small follicles with stagnant development, and they show a lower FSH threshold, which means a lower level of FSH stimulation is required for these patients to promote follicle regeneration and development (15). Our study demonstrated that PCOS was a high-risk factor for HOR, suggesting that PCOS patients are highly sensitive to gonadotropin. Furthermore, we also demonstrated a negative correlation between irregular menstrual cycles and POR, a condition that otherwise positively correlated with high response. Irregular menstrual cycle is one of PCOS diagnosis bases according to Rotterdam criteria (16). Our results further confirmed the positive correlation between PCOS and HOR. Endometriosis is another gynecological disease that severely endangers women's reproductive health. It was reported that endometriosis has a negative effect on ovarian reserve and response but shows little influence on pregnancy and live birth rate of IVF (17). Similarly, our present study also demonstrated the potential correlation of endometriosis and ovarian response, in which stages III and IV of endometriosis were positively related to POR, indicating that patients with the abovementioned two stages of endometriosis were more likely to show poor response. Additionally, our results also demonstrated that surgery history, especially pelvic-associated surgery including ovarian and fallopian tube operation, was positively correlated with ovarian response. Surgery for intraovarian diseases, such as ovarian cysts removal, in which electrocoagulation and hemostasis are applied on the ovarian wound, will directly destroy the ovarian tissue, causing damage to the ovarian function and ovarian reactivity. Concomitantly, clinical investigations have underpinned the concept that gynecological surgery is a pivotal factor impairing women ovarian reserve and response, as the consequence of deterioration in ovarian blood supply (18–20), which is intimately associated with the fallopian tube blood condition. Gynecological surgeries, especially salpingectomy, may affect the blood supply of ovary and subsequently lead to the downregulation of ovarian reserve and response. Indeed, our current study demonstrated surgery history was a risk factor of poor response.

It has been debated for decades whether the IVF cycles affect ovarian response. In clinical practice, the exogenous Gn is a widely used stimulatory drug to promote maximum recruitment and

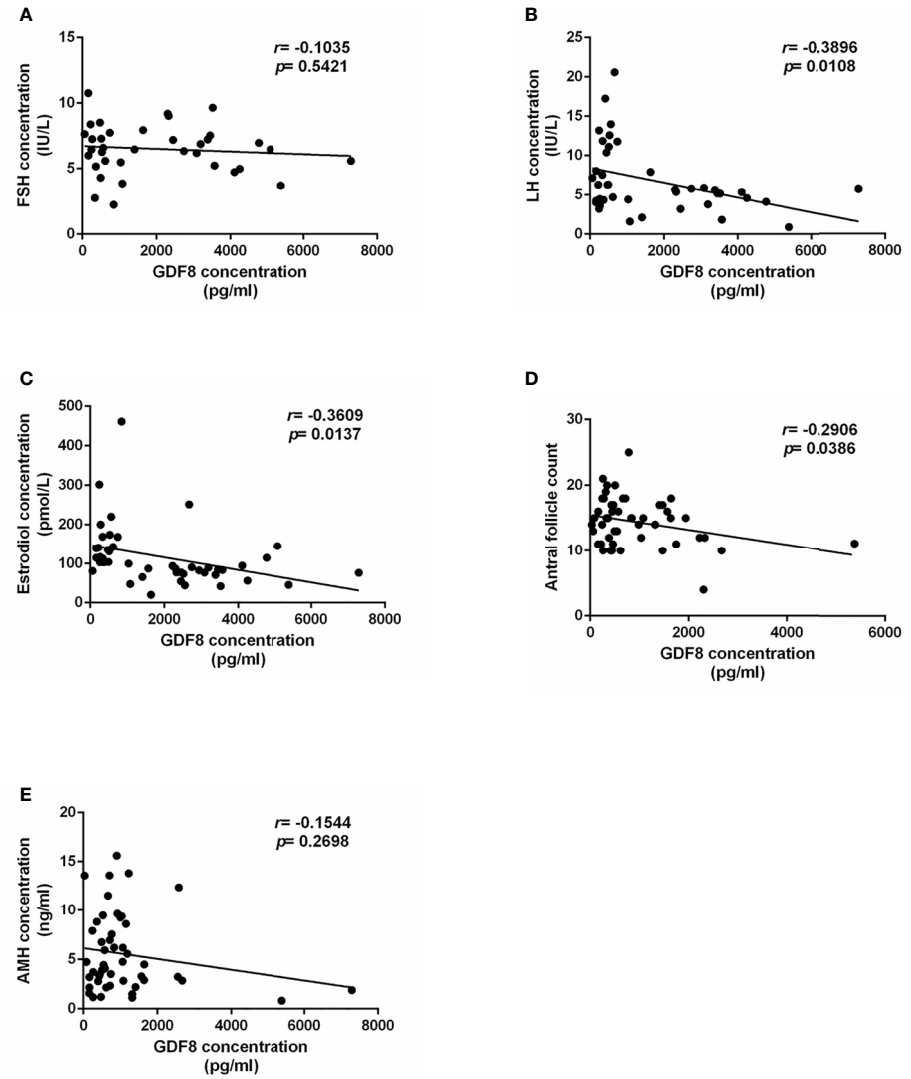

FIGURE 2 | The correlation analysis of GDF-8 and clinical ovarian response patients. **(A–C)** The correlation analysis of GDF-8 levels with hormone concentration, including FSH, LH, and estradiol. **(D)** The correlation analysis of GDF-8 levels with antral follicle count. **(E)** The correlation analysis of GDF-8 levels with AMH levels. The data were analyzed by the two-sample t test assuming unequal variances. $P < 0.05$ was considered statistically significant. n=51.

maturation of small follicles. Generally, the dose usage of Gn is often far beyond the normal physiological level, which accelerates the depletion of the primordial follicles, thus leading to the decline of ovarian reserve and response. This view is supported by a clinical case that a young infertile patient suffered from continuous decline of ovarian reserve after six consecutive IVF procedures within 4 years (21). Our present study suggested that four or more IVF cycles was a high-risk factor for POR.

The success of IVF-ET depends on the adequate follicle recruitment and maturation, achieved by the means of COH procedure. The proper ovarian response is a major determinant to the outcome of IVF-ET. POR would lead to less follicle recruitment and maturation, whereas HOR patients have an increased risk of developing ovarian hyperstimulation syndrome (OHSS) (5, 22). Our present study found a decrease in clinical pregnancy rates when it comes to patients with POR. Meanwhile, we also found that poor clinical outcomes are subjected to POR *via* imposing negative impact on the number of transplantable embryos and valid embryos. A cohort of investigations on COH patient has reported that HOR has no effect on quality of embryos, embryo implantation, and clinical pregnancy rate when compared to the normal ovarian response group (23, 24). In our study, we demonstrated that HOR population with <35 years old had a higher cancelation rate of IVF cycles, whereas a similar pregnancy rate compared with the control group, which was in accordance with the previous studies (23, 24). Meanwhile, we found a lower pregnancy rate in HOR patients with age ≥35. The diverse pregnancy outcomes in different ages are, at least in part, likely attributed to the changes of endometrial receptivity. The high estrogen levels

persistently stimulate the endometrium, leading to the insufficient endometrial secretory phase transformation and subsequently a decrease of pregnancy rate *via* inhibiting endometrial receptivity (25). Additionally, it should be claimed that our present study was based on the clinical information from a single clinical center. The large-scale investigation involved in multicenter clinical evaluation is needed to further confirm our results.

Cell–cell interactions and communications between oocyte and its supporting somatic cells play a crucial role in folliculogenesis. Oocyte and granulosa cell-derived cytokines and growth factors participate in follicle growth and maturation through autocrine/paracrine patterns (6). Multiple growth factors have been reported to regulate folliculogenesis. TGF-β is a well-studied growth factor superfamily encompassing more than 40 members. Within the ovary, TGF-β superfamily members are expressed in divergent cell types. AMH, a granulosa cell-derived growth factor, has been regarded as a reliable biomarker to predict the ovarian reserve and response (26). The studies on TGF-β superfamily member in ovarian physiology reveal the potential value of this functional superfamily in clinical application with an attempt to predict the ovarian response during COH process. Nevertheless, few studies have reported the possibility of other TGF-β superfamily members in evaluating ovarian response. In our current study, we explored the expression changes of GDF-8 in different ovarian response groups. GDF-8 levels were higher in POR groups, indicating GDF-8 was negatively correlated with POR. Importantly, we also demonstrated that the GDF-8 levels are negatively correlated with ovarian response, including LH and estradiol concentration, and antral follicle count. Our results provide a new insight on the possibility of GDF-8 regrading as a potential biomarker of the ovarian response during COH process. More recently, several studies have reported another dimension of the role of GDF-8 in modulating ovarian granulosa cell function (6, 11, 12). In particular, GDF-8 could influence the effects of gonadotropins by regulating their receptors expression (9). Meanwhile, the involvement of GDF-8 in PCOS has also been demonstrated, which underpins the function of GDF-8 in regulating folliculogenesis. In addition, the expression difference of responsible GDF-8 cellular receptors in granulosa cells were also investigated in our present study. Thus far, seven type I and five type II TGF-β superfamily receptors have been identified in mammalian cells. Once binding with the TGF-β superfamily member, the type I receptors will be phosphorylated and subsequently activate the downstream SMAD signaling pathway *via* inducing the phosphorylation. ALK5, ACVR2A, and ACVR2B have been identified as the targets of TGF-β type I and II receptors, mediating the function of GDF-8 in human granulosa cells (27, 28). Our results demonstrated that expression levels of ALK5 and ACVR2B in POR patients were higher compared with those in control groups. However, ACVR2A expression levels in POR patients were lower when compared to the control groups. Taken together, our results demonstrated a negative correlation between GDF-8, in concert with its responsible receptors, and POR. GDF-8 and its receptors appear to be the potential indicators for POR during COH.

In conclusion, our current study demonstrated the potential risk factors involved in different ovarian response during COH. Aging, obesity, endometriosis, surgery history, IVF treatment were the high-risk factors of POR, while irregular menstrual cycles and PCOS were the high-risk factors of HOR. Furthermore, POR patients had a decreased number of MII oocytes and available embryos, resulting in a lower clinical pregnancy rate. The levels of GDF-8, ALK5, and ACVR2B in POR patients were higher compared with those in control groups, whereas ACVR2A expression levels in POR patients were lower. Our study offers a new insight of risk factors correlated with ovarian response and highlights the potential role of GDF-8 level in indicating the ovarian response during COH process.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of the Women's Hospital, Zhejiang University School of Medicine, China. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LB, XY, and YMZ conceived the project and designed the experiments. LB and HHP performed and analyzed the bulk of the experiments. QQC and YJZ performed collection of clinical sample and patient information. YJZ and YX helped perform the experiments. LB and HHP wrote the manuscript, and XY and YMZ revised it. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.708089/full#supplementary-material

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A Proper Increasing in the Testosterone Level May Be Associated With Better Pregnancy Outcomes for Patients With Tubal or Male Infertility During *in vitro* Fertilization/Intracytoplasmic Sperm Injection

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We aimed to investigate the relationship between testosterone (T) levels and pregnancy outcomes in patients with tubal or male infertility at different times during *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (ICSI) cycles. Patients with tubal or male infertility and normal androgen levels undergoing IVF/ICSI were consecutively recruited. We performed a longitudinal analysis of T levels at three time points (i.e., T0: baseline, T1: trigger day, and T2: day after the trigger day) in three groups with different pregnancy outcomes (i.e., group 1: no pregnancy; group 2: clinical pregnancy but no live birth; and group 3: live birth) as repeated measurement data using linear mixed-effects models. We also plotted fitted curves depicting the relationship between T levels and a number of oocytes retrieved at different time points and identified the inflection points of the curves. In total, 3,012 patients were recruited. Groups 1 and 3 had improvements in T levels at the three time points. After refitting, the slope in group 3 was significantly higher than that in group 1 (P = 0.000). Curves that reflected the association between T levels and numbers of retrieved oocytes presented an upward trend before a certain inflection point, after which the curves had no obvious changes or fell with increasing T levels. The inflection points for T0, T1, and T2 were calculated as 0.45, 0.94, and 1.09, respectively. A faster upward trend in T levels might be associated with better pregnancy outcomes. Within a range lower than a T level inflection point, more oocytes and embryos could be obtained with increasing T levels.

Keywords: androgen, testosterone, *in vitro* fertilization (IVF), live birth rate, intracytoplasmic sperm injection (ICSI)

INTRODUCTION

Androgens, a category of sex steroid hormones, play an essential role in the endocrine and reproductive systems of women. The androgens that can be detected in the blood circulation of females include testosterone (T) (Gougeon, 1996), dihydrotestosterone, and pro-androgens such as dehydroepiandrosterone (DHEA) sulfate, DHEA, and androstenedione. These hormones

activate and exert effects on sensitive tissues *via* the androgen receptor of females, and T serves as the precursor for estradiol (E2) production (Simpson et al., 2002). In recent years, the physiology of androgen in females, which has complex effects on fertility, and its utilization in assisted reproductive technology (ART), has attracted interest from gynecologists (Simpson et al., 2000). Accumulating evidence from basic discovery research, clinical trials, and meta-analysis supports the hypothesis that androgens may have a synergistic stimulatory role with the follicle-stimulating hormone (FSH) in early follicle growth, follicle health maintenance, and follicle maturation during later stages of development (Meldrum et al., 2013).

Androgen levels, including that of T and DHEA, gradually decline with age among females aged 25–40 years (Davison et al., 2005). The addition of T or DHEA in females with poor response to recombinant FSH-induced ovarian stimulation during the *in vitro* fertilization (IVF) process has been broadly undertaken by medical centers (Gleicher and Barad, 2011). In contrast, high levels of androgens can prevent follicle maturation and even harm follicle development. Hyperandrogenism, such as polycystic ovarian syndrome (PCOS) and congenital adrenal hyperplasia, is a significant cause of female infertility (Dumesic et al., 2015). Studies have shown that excess androgen has a detrimental impact on fecundity (Mannerås et al., 2007). Excessive androgens can cause hyper-recruitment of follicles in the ovaries, leading to impaired maturation and infertility (Walters et al., 2019). The effect of androgens on follicle maturation and pregnancy outcome varies with their levels; therefore, evaluation of the association between androgens and IVF outcomes has clinical value. According to previous studies, basal T levels might be related to ovarian response competence and IVF outcomes. However, T levels at different time points during IVF cycles have not yet been reported. Therefore, we designed this retrospective study and investigated the relationship between T levels and pregnancy outcomes in patients with tubal or male infertility during different time points in the IVF/intracytoplasmic sperm injection (ICSI) cycles. It is noted that different indexes of androgens, including total T, androstenedione, and free androgen index (FAI), have been proved to be feasible indicator of hyperandrogenism in clinical practice, among which FAI had the best performance (Barth et al., 2010). In this retrospective study, we chose total T levels for analysis due to technical limitation in previous years. By exploring the changes in T levels during the stimulation cycle, we aimed to identify the optimal T levels during ovarian stimulation cycles and provide clinical evidence for adding androgens in patients with poor ovarian response (POR) during the IVF process. We also expected to provide appropriate target values for androgen-lowering regimens before IVF in patients with infertility and hyperandrogenism.

MATERIALS AND METHODS

Recruitment of Participants

The Institutional Review Board of the Peking Medical College Hospital (PUMCH) approved this retrospective observational study (No. S-K829). From July 2014 to March 2018, patients with tubal or male infertility and normal androgen levels undergoing IVF/ICSI at the PUMCH were consecutively recruited in this study. Written informed consent was obtained from all patients. Patients with hyperandrogenism, including PCOS and congenital adrenal hyperplasia, were excluded from this study. Other exclusion criteria were endometriosis; other endocrine disorders such as diabetes, pituitary dysfunction, or thyroid diseases, and a history of malignancy.

Baseline Clinical Characteristics and Hormonal Assays

The clinical characteristics of each patient during the IVF baseline were recorded, including age, body mass index (BMI), duration of infertility, methods of ART, infertility type, gestation history, types of controlled ovarian hyperstimulation (COH) protocol [gonadotropin-releasing hormone antagonist (GnRH-a) long protocol, GnRH-a ultra-long protocol, GnRH-a short protocol, GnRH antagonist protocol, and mini-stimulation protocol], and dosage of recombinant FSH (r-FSH) and human menopausal gonadotrophin (HMG).

At three time points during the IVF/ICSI cycles, T levels were regarded as the main variables for analysis. The three time points for testing the T level are listed as follows. First, we tested the T levels on the 2nd day of menstruation before COH as the baseline values, marked as T0. Second, T levels were measured on the trigger day when the patient received human chorionic gonadotropin (hCG) for final oocyte maturation, marked as T1. The third test, marked as T2, was performed the day after hCG injection.

Baseline hormone profiles, including human FSH, E2, luteinizing hormone (LH), and prolactin (PRL), were tested on the 2nd day of the menstrual cycle. Serum levels of hormones were measured using an electrochemiluminescent immunoassay (automated Elecsys Immunoanalyzer, Beckmann, United States). The mean interassay coefficients of variation were $< 5\%$ for T, $< 5\%$ for E2, and $< 8\%$ for FSH, LH, and PRL.

Confirmation of Primary and Secondary Outcomes

The primary outcomes of this study were clinical pregnancy rate and live birth rate. Clinical pregnancy was defined as the validation of the gestational sac and fetal heart using transvaginal ultrasound. Live birth was defined as the delivery of an infant born alive after 28 weeks of gestation. The cumulative outcomes within all the stimulation cycles of individual patients were evaluated in determining clinical pregnancy and live birth. The number of retrieved oocytes, metaphase II oocytes (MII), top-quality embryos (TQEs) on the 3rd day, and blastocyst-stage embryos were referred to as the secondary analysis outcomes. The definition of TQE was seven or more blastomeres, equally-sized blastomeres, and $< 20\%$ fragmentation on day 3 (Gardner and Schoolcraft, 1999). For patients who chose the freeze-all strategy after oocyte retrieval for various reasons, such as ovarian hyperstimulation syndrome risk and inflammation,

the cryopreserved blastocysts were thawed and subsequently transplanted. The cumulative live birth rate was likewise assessed.

Statistical Analyses

Continuous variables are described as mean ± SD, and categorical variables are expressed as percentages (%). The Student's *t*-test was used to compare continuous variables, and Fisher's exact test was used for categorical variables.

First, we conducted a longitudinal analysis of T levels within the cycle as repeated measurement data. The repeated measures analysis is used to examine response outcomes obtained from the same experimental unit at several time points. Longitudinal data are a typical kind of repeated measurement in which measurements are taken over time on specific individuals (Maurissen and Vidmar, 2017). Owing to the within-participant correlation of these data, linear mixed effect models were constructed using random intercept random slope models for analysis. The restricted maximum likelihood (MLE) method was used to refit the models to a straight line and calculate the regression estimates and 95% confidence intervals of the linear mixed-effects models. We performed repeated measurement analysis using a module in R (R Foundation for Statistical Computing, Vienna, Austria).[1] The module was designed to examine the association between the risk factor (*X*) and the outcome variable (*Y*) using linear mixed effect models, where a smooth fitting curve could be specified and a random effect could be introduced. The data for the module generally had a time variable (*T*), outcome variables varying with time, while the risk factor (*X*) in turn might have an influential effect on the outcome variable (*Y*). In our analysis, we identified the T level change as *Y*, the groups with different pregnancy outcomes as *X*, and the different time points for T level examinations as the time variable.

To further identify the possible relationship of T levels at different time points with pregnancy outcomes, we plotted smooth fitting curves to fit the T levels at different time points and secondary pregnancy outcomes (i.e., the number of oocytes retrieved, metaphase II oocytes, TQE at day 3, and blastocyst-stage embryos) using the generalized additive model-based spline smoothing method, adjusting for possible-related factors (i.e., age, BMI, methods of ART, duration of infertility, infertility type, gestation history, types of COH protocol, and dosage of recombinant r-FSH and HMG) as cofounders. To further identify the inflection points of the fitted curves, we then applied segmented regression, known as piece-wise regression, to fit each interval using a distinct line segment. The log-likelihood ratio test was used to assess if a threshold exists by comparing a one-line (non-segmented) model with a segmented regression model. Statistical significance of segmented linear regression with break-point was determined using variance analysis and *F*-tests. The β coefficients of the two segments before and after the inflection point were calculated using the effect-size metric. The differences in the slopes between the two segments were evaluated using the Wald test.

For sample size estimation, we first calculated that the ratio of T level changes from T0 to T2 in the patients with no pregnancy and live birth were 0.62 and 0.50, respectively. The ratio of the two groups was 0.97. When applying the sample size of 3,012, above 95% power could be obtained with a 5% two-sided significance.

Statistical analyses were performed using R (see text footnote 1) and EmpowerStats software 2.2 (X&Y solutions, Inc., Boston, MA). Statistical significance was set at $P < 0.05$.

[1] http://www.R-project.org

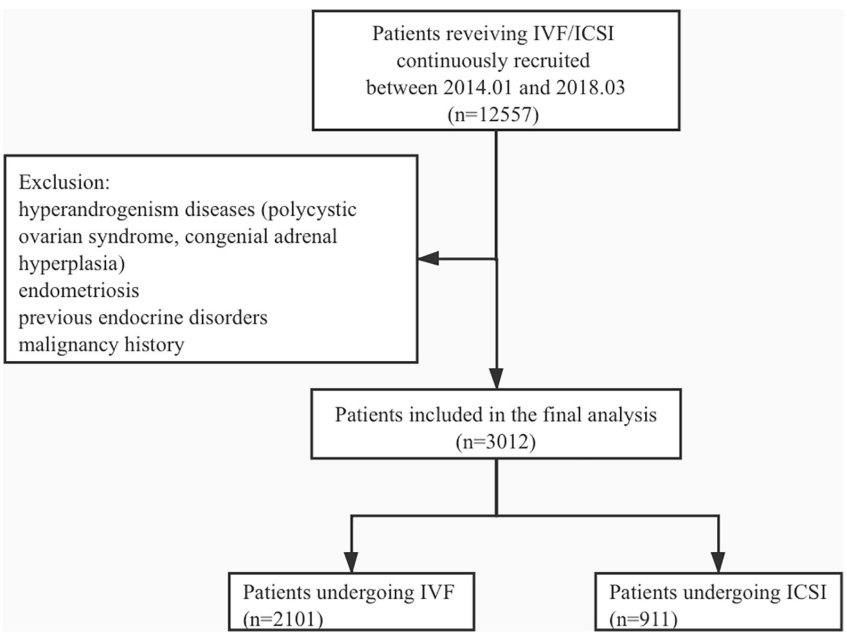

FIGURE 1 | The schematic of the study flowchart.

RESULTS

Patient Characteristics and Hormone Testing

A total of 3,012 patients undergoing IVF/ICSI were recruited for this study. The mean age of patients was 34.9 ± 4.3, and a total of 2,101 patients underwent IVF cycles, whereas 911 underwent ICSI. The study flowchart is shown in **Figure 1**. We divided the patients into three groups according to pregnancy outcomes as follows: group 1, no clinical pregnancy; group 2, clinical pregnancy but no live birth; and group 3, live birth. The clinical characteristics of patients from the three groups are shown in **Table 1**.

Longitudinal Analysis of the Repeated Measurement of T Levels at Three Time Points

The changes in T levels among the three groups at the baseline, trigger day of hCG administration, and the day after hCG uptake are illustrated in **Figure 2**. **Table 2** describes the results of a comparison of T levels at different time points. For groups 1 and 3, the lines presented an overall upward trend, and improvements in T levels were observed over time [group 1: P(T1 - T0) = 0.000 and P(T2 - T0) = 0.0001; group 3: P(T1 - T0) = 0.000 and P(T2 - T0) = 0.000]. The slope of T0 - T2 in group 3 after refitting using the MLE method was significantly higher than that in group 1 (P = 0.000), indicating that the upward trend of T levels in live births was significantly faster than that among participants with no clinical pregnancies.

Fitted Curves on the Relationship Between T Levels and Pregnancy Outcomes

We plotted the three fitted curves to illustrate the association between T levels and the number of retrieved oocytes at the three time points (T0, T1, and T2) (**Figure 3**). In the beginning, all curves had an upward trend, and after a certain inflection point, the curves showed no obvious changes or fell with increasing T levels. The inflection points for T0, T1, and T2 were calculated as 0.45, 0.94, and 1.09, respectively. The differences in the slopes before and after the inflection points were significant for the three curves [P(T0) = 0.0480, P(T1) < 0.0001, and P(T2) < 0.0001] (**Table 3**).

According to these results, we can conclude that at the baseline, the number of retrieved oocytes increases with T levels when the T level was < 0.45 ng/ml but was not associated with the T levels when the T level was > 0.45 ng/ml. On the trigger day, the number of retrieved oocytes increased with T levels when the T level was < 0.94 ng/ml but was not associated with the T levels when the T level was > 0.94 ng/ml. On the day after hCG administration, the value of T level is 1.07 when the numbers of retrieved oocytes start to decline with increasing T. The results of the comparison of pregnancy outcomes between patients with T levels lower than and higher than the inflection points during the three time points are shown in **Table 4**. Significant differences were detected in the secondary outcome variables, indicating that T levels higher than the inflection point during the three time points were associated with more acquired oocytes and embryos.

The fitted curves and inflection point calculations of the T levels and other outcomes, including numbers of metaphase II

TABLE 1 | Baseline characteristics of participants.

	G1: No clinical pregnancy	G2: Clinical pregnancy	G3: Live birth	*P*-value
N	1,427	204	1,381	
Age (years)	35.33 ± 4.51	35.26 ± 4.10	34.43 ± 4.13	<0.001
BMI (kg/m^2)	21.98 ± 3.18	22.40 ± 3.32	22.16 ± 3.11	0.102
Gravidity	1.15 ± 1.57	1.04 ± 1.32	0.86 ± 1.26	<0.001
Parity	0.11 ± 0.34	0.11 ± 0.32	0.07 ± 0.26	<0.001
Duration of infertility (years)	4.82 ± 3.26	5.05 ± 3.43	4.56 ± 2.79	0.025
Types of infertility				0.146
Primary infertility	804 (56.35%)	108 (53.20%)	817 (59.16%)	
Secondary infertility	623 (43.65%)	96 (46.80%)	564 (40.84%)	
Method				0.389
IVF	990 (69.45%)	151 (74.02%)	960 (69.44%)	
ICSI	437 (30.55%)	53 (25.98%)	421 (30.56%)	
Basal FSH (IU/L)	8.18 ± 4.03	7.74 ± 3.08	7.60 ± 3.33	<0.001
Basal LH (IU/L)	4.40 ± 3.55	4.12 ± 2.11	4.21 ± 2.67	0.166
Basal PRL (ng/ml)	17.92 ± 9.50	18.63 ± 10.19	17.82 ± 8.62	0.503
Basal E2 (pg/ml)	50.66 ± 31.17	49.93 ± 25.82	51.55 ± 34.40	0.675
Basal T (ng/ml)	0.48 ± 0.78	0.56 ± 1.49	0.45 ± 0.43	0.104
Total consumption of r-FSH (dose)	36.11 ± 12.60	32.72 ± 9.84	30.16 ± 16.11	<0.001
Total consumption of HMG (dose)	5.19 ± 3.71	5.08 ± 3.89	4.95 ± 4.05	0.002
COH protocol				0.009
GnRH-a long protocol	896 (62.79%)	127 (62.25%)	960 (69.51%)	
GnRH-a ultra-long protocol	30 (2.10%)	7(3.43%)	37 (2.68%)	
GnRH-a short protocol	2 (0.01%)	0 (0.00%)	2 (0.14%)	
GnRH-ant protocol	496 (34.76%)	70 (34.31%)	382 (27.66%)	
Mini-stimulation protocol	3 (0.21%)	0 (0.00%)	0 (0.00%)	

BMI, body mass index; IVF, in vitro fertilization; ICSI, intracytoplasmic sperm injection; FSH, follicle-stimulating hormone; E2, estrogen; LH, luteinizing hormone; PRL, prolactin; T, testosterone; GnRH-a, gonadotropin-releasing hormone agonist; GnRH-ant, gonadotropin-releasing hormone antagonist; COH, controlled ovarian hyperstimulation; r-FSH, recombinant FSH; HMG, human menopausal gonadotrophin.

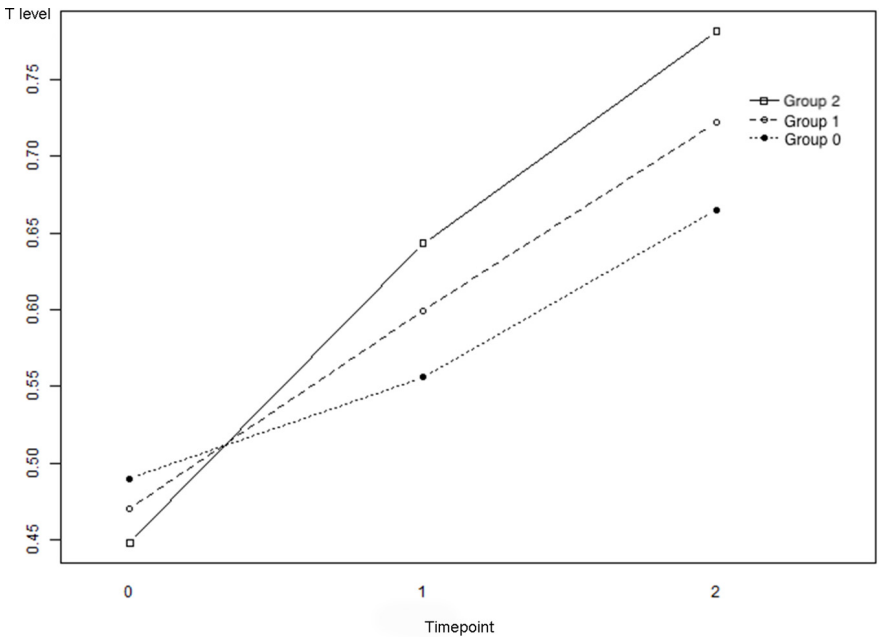

FIGURE 2 | The changes of the testosterone (T) levels of Groups 1–3. Group 1: no clinical pregnancy; Group 2: clinical pregnancy and no live birth; and Group 3: live birth. T0: T levels at baseline, T1: T levels on the trigger day, and T2: T levels on the day after the trigger day.

TABLE 2 | Repeated measurement analysis of androgen levels at three time points with different IVF outcomes using linear mixed effect models.

	T0(ng/ml)	T1(ng/ml)	T2(ng/ml)	P*	T1-T0		T2-T0		Refitting	
					B^a	P	B^b	P	B^c	P
G1 (*n* = 1,427)	0.48 ± 0.78	0.60 ± 0.25	0.72 ± 0.38	0.104	**0.0664 ± 0.0165**	**0.000**	**0.175 ± 0.0165**	**0.000**	/	/
G2 (*n* = 204)	0.56 ± 1.49	0.58 ± 0.24	0.71 ± 0.28	0.644	0.0626 ± 0.0467	0.179	0.0773 ± 0.0467	0.098	0.0270 ± 0.0241	0.262
G3 (*n* = 1,381)	0.45 ± 0.43	0.60 ± 0.23	0.73 ± 0.33	0.674	**0.129 ± 0.0234**	**0.000**	**0.159 ± 0.0234**	**0.000**	**0.0542 ± 0.0121**	**0.000**

**P-value of comparing androgen levels at three time points.*
[a]Regression estimate of T1 - T0 changes, values in bold have statistical significance.
[b]Regression estimate of T2 - T0 changes, values in bold have statistical significance.
[c]Regression estimate of comparing the refitting lines of G1 with G0 and G2 with G0, values in bold have statistical significance.
T, testosterone.
T0: T levels at baseline, T1: T levels on the trigger day, and T2: T levels on the day after the trigger day.

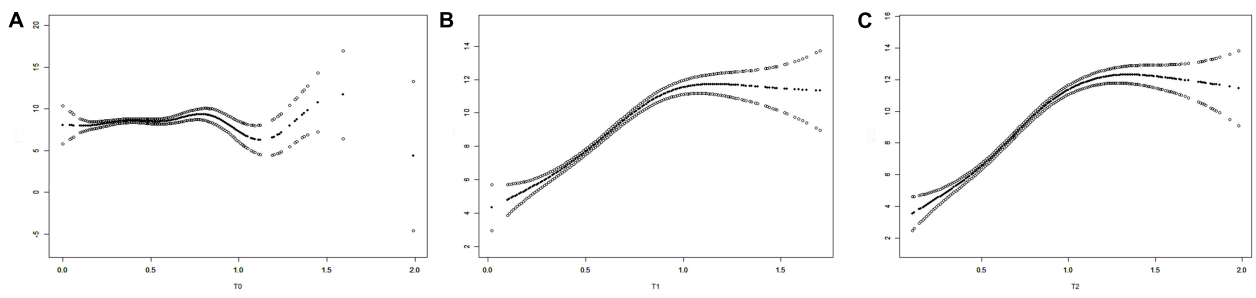

FIGURE 3 | The fitting curves showing the relationship of the T levels and the numbers of oocytes retrieved [**(A)** T0, **(B)** T1, and **(C)** T2). T0: T levels at baseline, T1: T levels on the trigger day, and T2: T levels on the day after the trigger day.

(MII) oocytes, numbers of TQEs, numbers of blastocyst-stage embryos, TQE formation rate, and blastocyst formation rate, are shown in **Supplementary Figures 1–5**. The fitting curves presented a similar trend with that of T levels and oocytes retrieved with corresponding inflection points, except for that of T levels and TQE formation rate.

TABLE 3 | The analysis of the inflection points and the effect sizes of the curves reflecting the association of T levels and numbers of oocytes retrieved.

	T0	T1	T2
Inflection point (K) (ng/ml)	0.45	0.94	1.09
β1 (95%CI) < k*	1.86 (0.09, 3.63)	8.92 (7.96, 9.68)	9.65 (8.96, 10.34)
P (β1)	0.0390	<0.0001	<0.0001
β2 (95%CI) > k**	0.03 (−0.21, 0.28)	−0.37 (−2.52, 1.78)	−1.42 (−2.20, −0.65)
P (β2)	0.7980	0.7352	0.0003
Difference for (β2-β1)	−1.83(−3.64, −0.02)	−9.19 (−11.76, −6.62)	−11.07 (−12.23, −9.91)
P (β2-β1)	0.0480	<0.0001	<0.0001

*β coefficient of the section before the inflection point.
**β coefficient of the section after the inflection point.
T, testosterone.
T0: T levels at baseline, T1: T levels on the trigger day, and T2: T levels on the day after the trigger day.

DISCUSSION

In this study, we found that for patients with tubal or male infertility who underwent IVF/ICSI, the cumulative live birth rate was higher among those who had a faster T level upward trend from the baseline to the trigger day. By examining the relationship between T level changes and the numbers of retrieved oocytes, we found that the highest level of oocyte retrieval rates can be acquired when the T levels reach 0.45 ng/ml at the baseline, 0.94 ng/ml on the trigger day, and 1.09 ng/ml on the day after hCG administration. Therefore, we hypothesized that a proper increase in T levels during ovarian hyperstimulation might increase the number of retrieved oocytes and have a positive impact on IVF outcomes.

In healthy females, androgens are a category of essential hormones that are highly involved in the promotion of follicular development by enhancing follicle recruitment and growth (Vendola et al., 1998), as well as increasing insulin-like growth factor 1 expression in the ovary (Vendola et al., 1999). Some animal studies have also shown that androgens are beneficial in follicular development through their promotion of preantral and small antral follicles in a dose-dependent manner (Shorakae et al., 2014; Lebbe et al., 2017). There is also clinical evidence indicating that androgen levels are positively correlated with ovarian response and may predict IVF outcomes (Luo et al., 2014; Sun et al., 2014). In contrast, the overexpression of androgens in patients with PCOS and other hyperandrogenic diseases can induce adverse effects on the preovulatory follicles, leading to anovulation and infertility (Dilaver et al., 2019; Owens et al., 2019). Androgens are also likely to play a role in the success rate of IVF in terms of their double-edged impact on follicle development and fertility. With the increasing use of androgen pretreatment to improve the ovarian response to hyperstimulation in patients with POR, a comprehensive assessment of the role of androgens in females undergoing IVF is needed.

The POR is a major cause of IVF failure. The addition of exogenous androgens or androgen-modulating agents in patients with POR who are undergoing IVF has been broadly utilized in clinical settings (Montoya-Botero et al., 2019). Recent studies have also focused on the pretreatment effects of androgens in patients with POR before undergoing IVF. However, contradictory results have been reported; some studies confirmed the efficacy of androgens in enhancing the live birth rate (Bosdou et al., 2016; Doan et al., 2017; Saharkhiz et al., 2018), whereas some negated this conclusion (Sipe et al., 2010; Bosdou et al., 2016). This contrast could be partly accounted for by the differences in study populations and the timing and duration of androgen pretreatment. In addition, the androgen level changes after the addition of androgens, and the association between androgen changes and IVF outcomes have not been explored.

For females undergoing IVF without endocrine abnormalities, the role of T levels in predicting IVF outcomes has not yet been established. One study on basal T levels in females with normal ovarian reserve indicated that a low T level might be relevant to the inadequate ovarian response during IVF (Qin et al., 2011). John et al. also suggested that a T level ≤ 20 ng/dl might be correlated with poor IVF outcomes, but other studies have refuted the predictive role of T (Barbieri et al., 2005; Walters et al., 2008). Furthermore, changes in androgen levels were not discussed in these studies.

TABLE 4 | Comparisons of pregnancy outcomes between patients with T levels ≤ and > the inflection point at the three time points.

	T0			T1			T2		
Testosterone (ng/ml)	**≤0.45**	**>0.45**	***P***	**≤0.94**	**>0.94**	***P***	**≤1.09**	**>1.09**	***P***
N	1,809	1,195		2,761	251		2,704	308	
No. of oocytes retrieved	8.22 ± 4.97	8.87 ± 5.15	<0.001	8.17 ± 4.92	11.96 ± 5.08	<0.001	8.06 ± 4.87	12.21 ± 5.04	<0.001
No. of MII oocytes	7.04 ± 4.71	7.65 ± 4.87	<0.001	7.00 ± 4.64	10.39 ± 5.10	<0.001	6.90 ± 4.58	10.61 ± 5.12	<0.001
No. of two-pronuclear zygotes	6.35 ± 4.65	6.88 ± 4.81	<0.001	6.31 ± 4.58	9.36 ± 5.26	<0.001	6.23 ± 4.52	9.50 ± 5.36	<0.001
No. of cleavage-stage embryos	6.31 ± 4.66	6.86 ± 4.82	<0.001	6.27 ± 4.60	9.36 ± 5.25	<0.001	6.19 ± 4.53	9.49 ± 5.36	<0.001
No. of TQE on the 3rd day	0.81 ± 1.27	0.84 ± 1.23	0.463	0.80 ± 1.24	1.10 ± 1.37	<0.001	0.77 ± 1.22	1.25 ± 1.50	<0.001
No. of blastocyst-stage embryos	1.66 ± 2.54	1.95 ± 2.68	<0.001	1.65 ± 2.50	3.12 ± 3.22	<0.001	1.61 ± 2.45	3.21 ± 3.31	<0.001

T, testosterone, MII, metaphase II; TQE, top-quality embryo.
T0: T levels at baseline, T1: T levels on the trigger day, and T2: T levels on the day after the trigger day.

To the best of our knowledge, this is the first study to explore the relationship between IVF outcomes and the T changes at different time points in the IVF cycles. In this study, we found that patients with a faster change in T levels from baseline to trigger day were more likely to achieve good IVF outcomes. This study may also explain the differential treatment efficacy of androgen pretreatment in patients with POR, as changes in T levels might influence outcomes. We planned to investigate androgen changes with pregnancy outcomes in patients receiving androgen pretreatment before IVF through further prospective cohort studies. Based on the results of this study, a reference goal of T reduction before undertaking IVF could be obtained for patients with hyperandrogenism.

This study has several limitations. It is noted that we did not use FAI for analysis, which had superior performance in determining hyperandrogenism for females than the total T according to Barth et al. (2010). Regrettably, the sex hormone-binding globulin (SHBG) examination has not been implemented regularly at our institution until 2020. As a consequence, in this study, the majority of the patients did not get SHBG and FAI data, which constitute the main limitation of our investigation. Considering that total T was proved to have a relatively acceptable accuracy in representing the androgen level of females (Barth et al., 2010), in this study, we think it can be a feasible alternative for FAI. In addition, FAI has been introduced as an essential indicator in our subsequent prospective and perspective studies. Moreover, in this study, only early follicular phase FSH was used to evaluate the ovarian functional reserve of the patient. Anti-Müllerian hormone (AMH) and antral follicle count (AFC) were not included, which were also not tested for the patients in our institution during the recruiting time. Other limitations include the retrospective and single-center design of the study. As a retrospective study, selection and recall biases were inevitable. We attempted to minimize recall bias by adjusting confounding variables and extracted the data from a computerized database. Also, this single-center study had a limited number of patients and IVF cycles. In future studies, the sample size should be enlarged to further validate our conclusion. Finally, we excluded patients with hyperandrogenism and focused mainly on patients with tubal or male infertility. Therefore, studies on androgen changes in patients with endocrine disorders are necessary.

CONCLUSION

By exploring the changes in T levels during various time points of the IVF/ICSI cycles, we found that the faster upward trend of the T levels might be associated with better pregnancy outcomes. Moreover, pregnancy outcomes are positively associated with T levels, within a certain range. Therefore, a proper increase in T levels might be beneficial for enhancing ovarian responses and IVF outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The Institutional Review Board approved the retrospective observational study of Peking Medical College Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DZ collected and validated the patient data. ZC analyzed and interpreted the patient data and was a major contributor in writing the manuscript. ZS and QY supervised this study and revised the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.696854/full#supplementary-material

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