Effect of Flexible GnRH antagonist and long GnRH agonist protocols on follicular fluid levels of PlGF, AMH, oocyte's morphology, and other IVF/ICSI outcomes in polycystic ovary syndrome women

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Research Article

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Abstract

Background: Gonadotropin-releasing hormone (GnRH) analogues are commonly used in clinical practice to prevent premature luteinizing hormone (LH) surge during In-Vitro Fertilization/ Intra-Cytoplasmic Sperm Injection (IVF/ICSI) cycles. However, the impacts of the GnRH analogues on the follicular microenvironment in women with polycystic ovary syndrome (PCOS) are still not elucidated.

Settings: This study was performed at Orient Hospital, Damascus, Syrian Arab Republic, from December 2019 to August 2021.

Methods: In this interventional, prospective, parallel, non-randomized, open-label controlled clinical trial, a total of 75 PCOS women (Rotterdam criteria) were allocated to take either the long GnRH agonist protocol (n=53) or the flexible GnRH antagonist protocol (n=22). The trial was originally designed to be a randomized control trial. However, due to a lack in the drug supply during a certain point of the study duration, we used a non-random study design. Follicular fluid (FF) samples were collected on the retrieval day, and the FF levels of Placental growth factor (PlGF) and Anti-Müllerian hormone (AMH) were measured using ELISA Kits. Before being subjected to ICSI, the mature oocytes from both groups were morphologically assessed under an inverted microscope at 400x magnification. In addition, the embryological and clinical IVF/ICSI outcomes were detected.

Results: The two groups did not differ significantly at baseline characteristics. The FF PlGF levels were significantly lower, while FFAMH levels were insignificantly higher in the GnRH antagonist group (FF PlGF; GnRH agonist = 142.75 ± 51.48 pg/ml vs. GnRH antagonist = 117.70 ± 35.86 pg/ml; P value = 0.028). (FF AMH; GnRH agonist = 13.62 ± 15.25 ng/ml vs. GnRH antagonist = 16.93 ± 18.08 ng/ml; P value = 0.492). The stimulation duration was significantly lower in the GnRH antagonist group compared to the long-agonist one (GnRH agonist = 8.04 ± 0.81 days vs. GnRH antagonist = 7.64 ± 1.22 days; P value = 0.011). On the other hand, although the consumed dose of gonadotropin was lower in the antagonist group, the difference between the two groups was not statistically significant (GnRH agonist = 1868.40 ± 668.29 IUs vs. GnRH antagonist = 1779.55 ± 702.87 IUs; P value = 0.432). Similarly, the number of retrieved oocytes, MII oocytes, MI oocytes, GV oocytes, fertilized oocytes, and embryos obtained were lower in the GnRH antagonist group, but the differences between the two groups were not statistically significant. In addition, there were not any significant differences between the two groups in oocytes morphology or other embryological or clinical IVF/ICSI outcomes.

Conclusions: Flexible GnRH antagonist protocol and the long GnRH agonist protocol regulate the follicular microenvironment and angiogenesis differently in PCOS subjects. However, these differences have no influence on the oocyte's morphology or the IVF/ICSI clinical outcomes. Thus, since flexible GnRH antagonist protocol represents a more friendly and cost-effective protocol, it may be a better treatment choice for PCOS women undergoing IVF/ICSI.

Study registration: This trial was prospectively registered at clinicaltrials.gov site under registration number (NCT04727671).

Introduction

Preventing premature luteinizing hormone (LH) surge is a fundamental step during controlled ovarian hyper-stimulation (COH) of IVF/ICSI cycle, which is commonly accomplished using one of the Gonadotropin-releasing hormone (GnRH) analogues, GnRH agonists or GnRH antagonists [1]. Nevertheless, they follow different suppression patterns. GnRH agonists act as indirect suppressors of the pituitary GnRH receptors causing a transient flare-up phase, followed by a down-regulation of GnRH receptors [1], while GnRH antagonists directly and competitively occupy the GnRH receptors leading to a dose-related inhibition of gonadotropins secretion [2]. However, GnRH receptors are expressed both in pituitary and extra-pituitary tissues, including the ovary [3], which may allow the GnRH analogues to affect the ovarian function through paracrine/autocrine mechanisms. Nevertheless, it is unclear whether these effects are dependent on the type of GnRH analogues used.

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder among reproductive-aged females, with a worldwide prevalence of 5–20% [4, 5]. The principal manifestations of this syndrome are ovulatory dysfunction, hyperandrogenism, and polycystic ovarian morphology [5]. Besides, PCOS accounts for 90–95% of anovulation cases attending infertility clinics [6]. Although the pathophysiology of PCOS is still not fully understood, growing evidence suggests a pivotal role for angiogenic dysregulation [7]. PCOS ovaries exhibit higher vascularization and lower impedance to flow in ovarian stromal vessels compared to control [8–10], which may be arisen from the differences in the levels of ovarian angiogenesis regulators as PCOS women showed higher levels of vascular endothelial growth factor (VEGF) and lower levels of the soluble form of VEGF receptor-1 (sVEGFR-1) compared to control both in serum and follicular fluid samples [11, 12]. In addition, Based on the study of Tal et al. [13], follicular fluid placental growth factor levels (FF PlGF), unlike serum levels, are increased in PCOS subjects. PlGF and VEGF are angiogenic growth factors belonging to the vascular endothelial growth factor (VEGF) family, which contains VEGF-A (also known as VEGF), VEGF-B, VEGF-C, VEGF-D, and VEGF-E, that is known for its role in regulating vasculogenesis and angiogenesis [14]. On the other hand, the sVEGFR-1 acts as an anti-angiogenic factor by sequestering VEGF and PlGF and decreasing their free form availability [15]. VEGF is considered the fundamental member of the VEGF family, and most of the available researche on angiogenesis were interested in detecting its role more than the other VEGF family members. However, although data are still relatively limited, higher levels of circulating PlGF have been linked to a variety of pathological conditions such as metastatic breast cancer [16], leukemia [17], rheumatoid arthritis [18], systemic lupus erythematosus [19], metabolic syndrome [20], type II diabetes [21], coronary artery disease [22], and neovascular age-related macular degeneration [23]. In addition, growing evidence has shown an important role of PlGF in regulating the reproduction process, starting from ovulation [24] to placentation, implantation, and embryo development [25–28]. Consequently, imbalance in PlGF levels may lead to pregnancy complications like preeclampsia, giving birth of small for gestational age, preterm birth, and stillbirth [29–32]. These effects may arise from the pleiotropic effects of PlGF, as besides its proangiogenic activity, PlGF acts as an immune modulator by enhancing monocyte [33] and macrophages activation [34], inhibiting dendritic cells differentiation and maturation [35], and regulating uterine NK cells proliferation and/or differentiation [36]. In addition, it skews the type

1 T helper immune response to the Th2 phenotype [35]. PlGF is expressed in follicular fluids at higher levels compared to circulation, and FF PlGF levels correlate positively with the number of retrieved oocytes of IVF/ICSI cycles [13]. However, no previous study has investigated the impact of the GnRH analogues protocols on the FF PlGF levels either in the general IVF/ICSI population or in the PCOS one.

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS) or Müllerian inhibiting substance factor (MIF), is a member of the transforming growth factor (TGF)-β family. It was initially recognized for its role in the differentiation of the male reproductive tract by triggering the regression of the Müllerian ducts, the Anlagen of the oviduct, uterus, cervix, and the upper part of the vagina [37, 38]. However, proceeding studies demonstrated its pivotal role in regulation ovarian folliculogenesis during both FSH-independent stages, by inhibiting the recruitment of primordial follicles [39–41]; and FSH-dependent stages, by reducing the follicular sensitivity to FSH [42, 43] and suppressing estradiol (E2) production from the granulosa cells (GCs) [43, 44]. In follicles, GCs are the principal producer of AMH [45], but the production depends on the follicle development stage. It is initiated at low levels in primary follicles, reaches its highest levels at pre-antral and small antral follicles, and subsequently declines during the following follicular growth stages [46–48]. Elevated AMH levels were observed both in serum and FF samples of PCOS women compared to controls [49–53]. Although the cause of this elevation is still unknown, current evidence suggests that it may be a result of the increased number of pre-antral and small antral follicles seen in PCOS ovaries and the overproduction of AMH per GC [54, 55]. In addition, some studies reported an abnormality in AMH/AMHR regulation system in PCOS subjects [56–59]. Previously, Winkler et al. [60] demonstrated a reduction in the expression of AMH from GCs after treating them with a GnRH antagonist for 24 hours. On the contrary, the expression was increased from the GnRH agonist-treated GCs models. Similarly, Dong et al. [61] noticed a down-regulation in the expression of AMH mRNA and AMH protein from the GnRH antagonist-treated GCs, but not from the GnRH agonist-treated GCs or the (GnRH agonist + GnRH-antagonist)-treated GCs. However, in these studies, the cells were treated with the GnRH analogues after they were cultured, and the authors did not mention whether the samples were taken after COH using the GnRH agonist protocol or the GnRH antagonist one. Moreover, it was unclear whether they included/excluded PCOS subjects from the study criteria. To the best of our knowledge, no previous study investigated the effect of GnRH analogues during COH protocols on the follicular fluid levels of AMH (FF AMH) in PCOS subjects.

Selecting the best gametes is crucial for IVF/ICSI success. Thus, mature oocytes would usually be subjected to a morphological assessment before being injected with the chosen sperms using an inverted microscope to evaluate their cytoplasmic maturation and detecting the presence of certain extra and intracytoplasmic morphologic features [62, 63]. However, it is still unclear how the type of GnRH analogue used in the COH protocol would influence the oocyte morphology; since some reports showed an improvement in oocyte quality in the GnRH agonist group compared to the GnRH antagonist one [64, 65], while others support the GnRH antagonist side [62], and some found no differences between the two groups [66, 67]. Nevertheless, none of these studies focused on the PCOS population.

Considering the previous data, we conducted this trial to compare the effect of the type of the GnRH analogues used in COH protocol on the follicular microenvironment of PCOS women undergoing IVF/ICSI cycles and to clarify whether these effects would have any impacts on the final IVF/ICSI embryological or clinical outcomes.

Objectives

This clinical trial aimed to compare the effects of the long GnRH agonist protocol and the flexible GnRH antagonist protocol on the follicular fluid levels of PlGF and AMH, clinical and embryological IVF/ICSI outcomes, including oocyte morphology in PCOS subjects.

Material And Methods

Study Design

This interventional, prospective, parallel, non-randomized, open-label controlled clinical trial was conducted on PCOS women who were referred to the Assisted Reproductive Unit of Orient Hospital, Damascus, Syrian Arab Republic, from December 2019 to August 2021. The Ethical Committee of Damascus University approved the study protocol, and a written informed consent was obtained from all participants. The clinical trial registration number is NCT04727671. The TREND (Transparent Reporting of Evaluations with Nonrandomized Designs) guideline was used to ensure transparent reporting of this study.

The trial was originally designed to be a randomized control trial (RCT). However, due to the medical supplies' crisis in the Syrian Arab Republic, it was challenging to provide the GnRH antagonist (Cetrorelix, Cetrotide; Merck) during a certain period of the study duration, meanwhile supplying the GnRH agonist (Triptorelin, Decapeptyl; Ferring) was less troublesome. Thus, the randomization was broken, and more patients were recruited in the GnRH agonist arm.

Participants

In this study, a total of 75 PCOS women were allocated to take either the long GnRH agonist protocol (n = 53) or the flexible GnRH antagonist protocol (n = 22). Both the patients and the doctors were aware of the allocated arm. PCOS diagnosed was according to the Rotterdam criteria [68]; the presence of at least two of the following three criteria: (1) oligo or anovulation, (2) clinical and/or biochemical signs of hyperandrogenism, (3) polycystic ovarian morphology on ultrasound examination (defined as the presence of 12 or more follicles in each ovary measuring 2–9 mm in diameter and/or an ovarian volume > 10 ml) with the exclusion of other possible etiologies. Patients who aged ≥ 40 years; or were diagnosed with androgen-secreting tumors, Cushing's syndrome, congenital adrenal hyperplasia, hyperprolactinemia, thyroid disorders, epilepsy, diabetes mellitus, cardiovascular diseases, liver diseases, kidney diseases, cancer; or had any conditions that might affect IVF outcomes like endometriosis, uterine fibroids, hydrosalpinx, adenomyosis, or autoimmune diseases were excluded. Women with three or more previous

IVF failures, poor responders (Bologna criteria [69]), and those who were previously undergone unilateral oophorectomy were also excluded.

Controlled ovarian stimulation protocols:

Agonist Group (Long protocol):

The pituitary down-regulation in this group was carried out using 0.05–0.1 mg of Triptorelin acetate subcutaneously (SC) once daily from the mid-luteal phase (day 21) of the menstrual cycle until the ovulation triggering day. When the suppressive effect was obtained (E2 < 50 pg/ml, no cysts or follicles > 1 cm maximum diameter detected by ultrasound, endometrial thickness < 5 mm), ovarian stimulation was commenced with recombinant Follicle-Stimulating Hormone (r-FSH) and/or human Menopausal Gonadotropin (hMG), and the dose was adjusted according to the ovarian response, which was monitored by transvaginal ultrasound (Voluson ^{TM} E10, GE Healthcare Ultrasound, USA).

Antagonist Group (Conventional Flexible protocol):

The ovarian stimulation in this group was started with recombinant Follicle-Stimulating Hormone (r-FSH) and/or human Menopausal Gonadotropin (hMG) on the third day of the menstrual cycle, and the dose was adjusted according to the ovarian response, which was monitored by transvaginal ultrasound (Voluson ^{TM} E10, GE Healthcare Ultrasound, USA). The initiation of 0.25 mg of GnRH antagonist, Cetrorelix, took place after detecting a leading follicle diameter ≥ 14 mm and continued till the day of ovulation triggering.

Ovulation triggering and oocytes retrieval:

Ovulation was triggered by the administration of 10,000 IU of Human Chorionic Gonadotropin (hCG) when at least three follicles become more than 16–17 mm. After 35 ± 2 hours of ovulation triggering, the oocytes were retrieved by transvaginal ultrasound-guided follicle aspiration.

IVF procedure and embryological outcomes assessment:

An Intra-Cytoplasmic Sperm Injection (ICSI) technique was used for insemination. The embryological outcomes were assessed by independent highly-trained embryologists. Each studied outcome was assessed by a single assessor for both groups to limit inter-assessor variations. The same media and culturing methodology were used for both groups. The Thermo Scientific HERACELL 150i incubator (Thermo Fisher Scientific, USA) was used for COCs and oocytes cultures (humidified atmosphere at 37ºC, CO2 level at approximately 6%, and culture medium pH between 7.28–7.35), and the K-Systems G210 InviCell (K-Systems Kivex Biotec Ltd. Denmark) was used for Embryos cultures.

Oocyte’s denudation and maturation assessment:

Retrieved oocytes were first rinsed in G-MOPS ^{TM} Plus media (G-MOPS ^{TM} Plus, Vitrolife, Sweden) then maintained in G-IVF ^{TM} Plus culture (G-IVF ^{TM} Plus, VitroLife, Sweden) covered with paraffin oil (OVOIL,

VitroLife, Sweden) before cumulus cell removal. The surrounding cumulus cells were removed within 2 hours after retrieval by the exposure to hyaluronidase (HYASE-10× in G-Mops ^{TM} Plus media, Vitrolife, Sweden) for several seconds before being transferred to G-MOPS ^{TM} Plus media where they were mechanically dissociated from the oocyte.

The denuded oocytes were classified according to their level of maturation using a Nikon SMZ1500 stereoscope. The number of Metaphase II Oocytes (MII; identified as oocytes with the extrusion of the first polar body), Metaphase I Oocytes (MI; identified as oocytes lack the presence of both the germinal vesicle and the polar body), Germinal Vesicle Oocytes (GV; identified as oocytes with Germinal Vesicle), and Atretic Oocytes (oocytes with signs of degeneration) were documented. The Maturation Rate was calculated by dividing the number of mature (MII) oocytes by the number of retrieved oocytes. In addition, the ovarian sensitivity index (OSI) was calculated by dividing the number of retrieved oocytes by the total dose of FSH used and multiplying the results by 1000 [70].

Oocytes morphological assessment:

Before being subjected to ICSI, MII oocytes from both groups were morphologically assessed using an inverted microscope Nikon Eclipse Ti2 (Nikon, Tokyo, Japan) under 400× magnification. The following dysmorphisms were studied:

- Cytoplasmic dysmorphisms: the presence of granulation, refractile bodies, smooth endoplasmic reticulum (SER) aggregations or vacuoles in the cytoplasm; or detecting dark cytoplasm.
- Extracytoplasmic dysmorphisms:
    - Alterations in oocyte shape or size.
    - Zona pellucida dysmorphisms: alterations in zona pellucida color, size, or thickness; the presence of a zona pellucida with a septum.
    - Perivitelline space dysmorphisms: alterations in perivitelline space size or presence of perivitelline space fragments.
    - Polar body dysmorphisms: alterations in polar body size, presence of polar body fragments, or presence of duplicated/triplicated polar body.

The oocytes were classified as normal oocytes, oocytes with cytoplasmic dysmorphisms, oocytes with extracytoplasmic dysmorphisms, and oocytes with both cytoplasmic and extracytoplasmic dysmorphisms. In addition, the oocytes were classified based on the quantity of the dysmorphisms observed.

Insemination and fertilization assessment:

Microinjections were performed at X400 magnification on a 37ºC heated stage inverted Nikon Eclipse Ti2 (Nikon, Tokyo, Japan). A Petri dish containing a microdroplet of ICSI ^{TM} media in the center (ICSI ™, VitroLife, Sweden) under paraffin oil (OVOIL, VitroLife, Sweden) was used for sperms selection and

immobilization. On the same dish, a microdroplet of G-Gamete ^{TM} culture medium (G-Gamete ™, Vitrolife, Sweden) was used for placing the oocytes for microinjection. A single sperm was mechanically immobilized using the tip of the microinjection needle (Origio, USA) and then was aspirated inside the needle. The oocyte was held in place using a 35-degree angle holding micropipette (Origio, USA) with the polar body in the 6 or 12 o'clock position. Injection of a single spermatozoon within the oocyte cytoplasm was performed by using a micromanipulator (TransferMan® 4r, eppendorf, Germany). After ICSI, injected oocytes were cultured in G1Plus ™ medium (G1-Plus ™, VitroLife, Sweden). Fertilization was confirmed by the presence of two pronuclei and the extrusion of the second polar body approximately 16–18 h after ICSI. The Fertilization Rate was calculated by dividing the number of obtained zygotes (2PN) by the number of injected oocytes.

Embryos Grading, Cleavage rate, and high-quality embryos rate:

Embryos were morphologically evaluated using Nikon SMZ1500 stereoscope microscope (Nikon, Tokyo, Japan) and were graded based on ESHRE criteria (2011) [71]. According to these criteria, high-quality cleavage-stage embryos are defined as those with all of the following characteristics: 2–4 cells on day 2 or 6–8 cells on day 3, < 10% fragmentation, symmetric blastomeres, and absence of multinucleation. Cleavage rate was calculated by dividing the number of cleavaged embryos by the number of obtained zygotes (2PN), while High-Quality Embryos Rate was calculated by dividing the number of high-quality embryos (Grade I) obtained by the total number of cleavaged embryos obtained.

Embryos transfer and luteal phase support:

The Selected embryos were treated with EmbryoGlue® media (EmbryoGlue®, VitroLife, Sweden) before being transferred using a Sure-Pro Ultra catheter (Wallace, USA) under transvaginal ultrasound guidance on day 2–3 after insemination (cleavage stage embryos). Luteal phase support was achieved using vaginal micronized progesterone gel (Crinone ® 8%, Merck Serono). It was started from the day of oocyte retrieval and continued for 14 days when a pregnancy was carried out. If pregnancy was confirmed, progesterone administration was continued until the 12th week of pregnancy.

Embryo transfer was cancelled, and elective embryo cryopreservation was performed in cases that were highly suspected of developing life-threatening (critical) OHSS [72, 73] or fulfilling the criteria for OHSS hospitalization [74]. Cycle Cancellation Rate (CCR) was calculated by dividing the number of cycle cancellation cases by the total number of participants.

Follicular fluid collection and analysis:

Follicular fluid was aspirated from all follicles (> 15) mm, and then it was centrifuged at 3000 g for 10 min at room temperature, and the supernatant was stored at − 80°C until assayed. Follicular fluid concentrations of AMH were assayed using an ELISA kit from Biorex diagnostics (United Kingdom). Follicular fluid concentrations of PlGF were assayed using an ELISA kit from DRG Instruments

(Germany). The intra-assay and interassay coefficients of variation for all assays were less than 5% and less than 10%, respectively.

Pregnancy assessment and follow up:

A serum pregnancy test (serum hCG) was performed 14 days after embryo transfer. All women with a positive test received a transvaginal ultrasound scan after one-two weeks (i.e., 3–4 weeks after embryo transfer) then followed up until week 12 of gestation. The following rates were calculated:

- Biochemical Pregnancy Rate (BPR): Biochemical pregnancy was defined as a positive serum beta-hCG pregnancy test after two weeks of embryo transfer [75]. BPR was calculated by dividing the number of women who were biochemically pregnant by the total number of participants (Per Woman) or the total number of women who had at least one embryo transferred (Per Embryo Transfer).
- Clinical Pregnancy Rate (CPR): Clinical pregnancy was defined as the presence of at least one gestational sac on ultrasound after 3–4 weeks of embryo transfer. In addition to intra-uterine pregnancy, it included a clinically documented ectopic pregnancy [75]. CPR was calculated by dividing the number of women who were clinically pregnant by the total number of participants (Per Woman) or the total number of women who had at least one embryo transferred (Per Embryo Transfer).
- Multiple Pregnancy Rate (MPR): MPR was calculated by dividing the number of pregnancies with two or more gestational sacs on ultrasound by the total number of participants (Per Woman) or the total number of women who had at least one embryo transferred (Per Embryo Transfer).
- Implantation Rate (IR): IR was calculated by dividing the number of gestational sacs observed by the number of embryos transferred.
- Ongoing Pregnancy Rate (OPR): Ongoing pregnancy was defined as a pregnancy that continued ≥ 12 weeks of gestation. OPR was calculated by dividing the number of ongoing pregnancies by the total number of participants (Per Woman) or the total number of women who had at least one embryo transferred (Per Embryo Transfer).
- Resolved Pregnancy of unknown location (RPUL) Rate: RPUL was defined as a pregnancy demise not visualized on transvaginal ultrasound with a resolution of serum β-hCG after expectant management or after uterine evacuation without chorionic villi on histology [76]. RPUL Rate was calculated by dividing the number of RPUL cases by the total number of participants (Per Woman) or the total number of women who had at least one embryo transferred (Per Embryo Transfer).

Statistical analysis

All statistical analyses were performed using a Statistical Package for the Social Sciences (SPSS) software version 24.0 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation and categorical variables as counts with percentages. Between-group comparisons were performed using the independent *t*-test for normally distributed variables, the Mann–Whitney *U* test

for non-normally distributed variables, and chi-square or Fisher's exact test as appropriate for categorical variables. For testing all hypotheses, tests were two-tailed, and values less than 0.05 were considered statistically significant.

Results

The two groups did not differ significantly in Female age, male age, infertility history, or other baseline characteristics, as shown in Table 1. The stimulation duration was significantly shorter in the GnRH antagonist group compared to the long-agonist one (GnRH agonist = 8.04 ± 0.81 days vs. GnRH antagonist = 7.64 ± 1.22 days; P value = 0.011; Table 2). On the other hand, although the consumed dose of gonadotropins was lower in the antagonist group, the difference between the two groups was not statistically significant (GnRH agonist = 1868.40 ± 668.29 IUs vs. GnRH antagonist = 1779.55 ± 702.87 IUs; P value = 0.432; Table 2). Four cycles were cancelled in the GnRH agonist group, of which two were due to high risk of developing severe OHSS, one was a hospitalized case for OHSS, and one was cancelled due to male factor as no embryos were obtained (the male has cryptozoospermia, and the female gave seven oocytes, of which four were MII oocytes, and three were MI oocytes. After ICSI, one zygote was obtained but did not develop into a cleavage embryo). On the other hand, three cycles were cancelled in the GnRH antagonist group, of which one was due to high risk of developing severe OHSS, and two were cancelled due to male factor as no zygotes were obtained (in one cycle, the male has cryptozoospermia and the female gave ten oocytes, of which nine were MII oocytes, and one was GV oocytes, while in the other cycle, the male has azoospermia and the female gave 29 oocytes, of which 18 were MII oocytes, 5 MI oocytes, and 6 GV oocytes). However, there were not any significant differences between the two groups in the total rate of cycle cancellation or in the rate of cancellation due to high risk of developing severe OHSS. Similarly, the other cycle characteristics did not differ significantly between the two groups, as shown in Table 2. Although the OSI and the number of retrieved oocytes, MII oocytes, MI oocytes, GV oocytes, fertilized oocytes, and embryos obtained were lower in the GnRH antagonist group, the differences between the two groups were not statistically significant (Table 3). Moreover, there were not any significant differences between the two groups in maturation rate, fertilization rate, highquality of embryos rate, and cleavage rate (Table 3). Interestingly, the FF PlGF levels were significantly lower in the GnRH antagonist group compared to the long-agonist one (GnRH agonist = 142.75 ± 51.48 pg/ml vs. GnRH antagonist = 117.70 ± 35.86 pg/ml; P value = 0.028; Table 3). On the other side, AMH levels were higher in the GnRH antagonist group, but the differences between the two groups were not statistically significant (GnRH agonist = 13.62 ± 15.25 ng/ml vs. GnRH antagonist = 16.93 ± 18.08 ng/ml; P value = 0.492; Table 3). In addition, the morphological assessment of MII oocytes showed comparable oocytes morphology in both groups. Regarding the clinical outcomes, both protocols achieved similar rates of biochemical pregnancy, clinical pregnancy, ongoing pregnancy, multiple pregnancy, implantation, resolved PUL, ectopic pregnancy, and hospitalized OHSS. Two women were hospitalized for severe OHSS in the GnRH against group; one developed OHSS before transferring the embryos. Thus, elective embryo cryopreservation was conducted, and the cycle was cancelled. The other

case developed severe OHSS 14 days after hCG. In the GnRH antagonist group, one woman was hospitalized due to moderate OHSS 15 days after hCG.

Table 1
Patients Baseline Characteristics.

	GnRH Agonist N = 53	GnRH Antagonist N = 22	P value
Female age (years)	27.87 ± 4.57	27.09 ± 5.15	0.411
Male age (years)	35.51 ± 6.41	34.64 ± 6.96	0.838
Infertility % (n):	67.9% (36/53)	54.5% (12/22)	0.272
Primary	32.1% (17/53)	45.5% (10/22)	
Secondary			
Infertility cause % (n):	18.9% (10/53)	27.3% (6/22)	0.195
PCOS only	81.1% (43/53)	68.2% (15/22)	
PCOS + Male factor	0.0% (0/53)	4.5% (1/22)	
PCOS + Tubal factor	0.0% (0/53)	0.0% (0/22)	
PCOS + Male factor + Tubal factor			
Infertility duration (years)	5.75 ± 3.35	6.25 ± 4.62	0.995
Smoker Female % (n)	18.9% (10/53)	36.4% (8/22)	0.106
Smoker Male % (n)	50.9% (27/53)	63.6% (14/22)	0.315
Female alcohol-consuming % (n)	0.0% (0/53)	0.0% (0/22)	-
Male alcohol-consuming % (n)	1.9% (1/53)	0.0% (0/22)	1.000
Male classification % (n):	13.2% (7/53)	36.4% (8/22)	0.284
Normozoospermia	32.1% (17/53)	31.8% (7/22)	
Mild-Moderate Male factor	32.1% (17/53)	18.2% (4/22)	
Oligoasthenoteratozoospermia	15% (8/53)	9.1% (2/22)	
Azoospermia	3.8% (2/53)	0.0% (0/22)	
Necrozoospermia	3.8% (2/53)	4.5% (1/22)	
Cryptozoospermia			
PCOS: Polycystic Ovary Syndrome.			

Table 2
Cycle Characteristics:

	GnRH Agonist N = 53	GnRH Antagonist N = 22	P value
Types of stimulator % (n):	88.7% (47/53)	81.9% (18/22)	0.579
r-FSH	1.9% (1/53)	4.5% (1/22)	
hMG	9.4% (5/53)	13.6% (3/22)	
r-FSH + hMG			
FSH starting dose (units)	227.83 ± 72.00	225.00 ± 83.45	0.690
Total FSH dose (units)	1868.40 ± 668.29	1779.55 ± 702.87	0.432
Stimulation duration (days)	8.04 ± 0.81	7.64 ± 1.22	0.011
Sperms Source % (n):	75.5% (40/53)	77.3% (17/22)	0.093
Ejection	22.6% (12/53)	9.1% (2/22)	
Tesa	0.0% (0/53)	0.0% (0/22)	
Pesa	0.0% (0/53)	4.5% (1/22)	
Frozen	1.9% (1/53)	9.1% (2/22)	
Ejection + Tesa			
Day of transfer	63.3% (31/49)	57.9% (11/19)	0.683
Day 2	36.7% (18/49)	42.1% (8/19)	
Day 3			
Cycle cancellation Rate % (n)	7.5% (4/53)	13.6% (3/22)	0.412
Cycle cancellation Rate due to risk of OHSS % (n)	5.7% (3/53)	4.5% (1/22)	1.000

hMG: human Menopausal Gonadotropin, OHSS: Ovarian Hyperstimulation Syndrome, Pesa: Percutaneous Epididymal Sperm Aspiration, r-FSH: recombinant Follicle-Stimulating Hormone, Tesa: Testicular Sperm Aspiration.

Table 3
Embryological IVF/ICSI Outcomes and oocyte morphology assessment:

	GnRH Agonist N = 53	GnRH Antagonist N = 22	P value
Number of Retrieved Oocytes	20.04 ± 9.42	17.73 ± 9.76	0.242
Ovarian Sensitivity Index	11.83 ± 6.98	11.46 ± 8.99	0.419
Number of Metaphase II Oocytes	11.89 ± 5.65	10.18 ± 5.55	0.235
Number of Metaphase I Oocytes	4.19 ± 3.23	3.55 ± 2.74	0.298
Number of GV Stage Oocytes	3.09 ± 2.31	2.5 ± 3.52	0.070
Number of Atretic Oocytes	0.87 ± 1.84	1.5 ± 2.76	0.623
Number of Fertilized Oocytes	7.42 ± 4.17	6.73 ± 4.78	0.513
Maturation Rate (%)	61.39 ± 14.30	60.13 ± 23.79	0.646
Fertilization Rate (%)	63.55 ± 23.55	69.18 ± 31.72	0.197
Number of Embryos Obtained	7.32 ± 4.07	6.68 ± 4.82	0.506
High-quality Embryos Rate (%)	56.83 ± 23.85	60.41 ± 30.62	0.360
Cleavage Rate (%)	97.61 ± 13.92	89.39 ± 29.79	0.225
Number of Embryos Transferred	4.47 ± 1.93	3.45 ± 2.41	0.111
FF AMH ng/ml	13.62 ± 15.25	16.93 ± 18.08	0.492
FF PlGF pg/ml	142.75 ± 51.48	117.70 ± 35.86	0.028
Oocytes Morphology % (n):	77.3% (41/53)	77.3% (17/22)	0.521
Normal	18.9% (10/53)	18.2% (4/22)	
Cytoplasmic Dysmorphisms	0.0% (0/53)	4.5% (1/22)	
Extra-Cytoplasmic Dysmorphisms	3.8% (2/53)	0.0% (0/22)	
Both			
Quantity of oocytes dysmorphisms % (n):	77.3% (41/53)	77.3% (17/22)	0.610
Normal	17.0% (9/53)	22.7% (5/22)	
One	5.7% (3/53)	0.0% (0/22)	
Multi			

AMH: Anti-Müllerian Hormone, GV: Germinal Vesicle, PB: Polar Body, PlGF: Placental Growth Factor, PVS: Perivitelline Space, SER: Smooth Endoplasmic Reticulum Aggregations, ZP: Zona Pellucida.

	GnRH Agonist N = 53	GnRH Antagonist N = 22	P value
Granulation % (n)	17% (9/53)	9.1% (2/22)	0.491
Refractile Bodies % (n)	0.0% (0/53)	0.0% (0/22)	-
SER % (n)	0.0% (0/53)	0.0% (0/22)	-
Vacuoles % (n)	5.7% (3/53)	9.1% (2/22)	0.627
Dark Cytoplasm % (n)	1.9% (1/53)	0.0% (0/22)	1.000
Oocytes Shape % (n)	0.0% (0/53)	4.5% (1/22)	0.293
Oocytes Size % (n)	0.0% (0/53)	0.0% (0/22)	-
ZP Dysmorphisms % (n)	0.0% (0/53)	0.0% (0/22)	-
PVS Dysmorphisms % (n)	0.0% (0/53)	0.0% (0/22)	-
PB Dysmorphisms % (n) (Duplicated/ Triplicated PB)	3.8% (2/53)	0.0% (0/22)	1.000

AMH: Anti-Müllerian Hormone, GV: Germinal Vesicle, PB: Polar Body, PlGF: Placental Growth Factor, PVS: Perivitelline Space, SER: Smooth Endoplasmic Reticulum Aggregations, ZP: Zona Pellucida.

Table 4
Clinical IVF/ICSI Outcomes:

	GnRH Agonist N = 53	GnRH Antagonist N = 22	P value
Endometrial thickness on hCG day (mm)	9.63 ± 1.19	9.71 ± 1.37	0.920
Biochemical Pregnancy Rate % (n)	43.4% (23/53)	36.4% (8/22)	0.573
Per Woman:	46.9% (23/49)	42.1% (8/19)	0.720
Per Embryo Transfer:			
Clinical Pregnancy Rate % (n)	39.6% (21/53)	36.4% (8/22)	0.792
Per Woman:	42.9% (21/49)	42.1% (8/19)	0.955
Per Embryo Transfer:			
Ongoing Pregnancy Rate % (n)	32.1% (17/53)	36.4% (8/22)	0.720
Per Woman:	34.7% (17/49)	42.1% (8/19)	0.570
Per Embryo Transfer:			
Multiple Pregnancy Rate % (n)	17% (9/53)	22.7% (5/22)	0.536
Per Woman:	18.4% (9/49)	26.3% (5/19)	0.512
Per Embryo Transfer:			
Implantation Rate %	15.06 ± 22.86	18.05 ± 31.14	0.926
Resolved PUL% (n)	3.8% (2/53)	0.0% (0/22)	1.000
Per Woman:	4.1% (2/49)	0.0% (0/19)	1.000
Per Embryo Transfer:			
Ectopic Pregnancy Rate % (n)	3.8% (2/53)	0.0% (0/22)	1.000
Per Woman:	4.1% (2/49)	0.0% (0/19)	1.000
Per Embryo Transfer:			
Hospitalized OHSS Rate % (n)	3.8% (2/53)	4.5% (1/22)	1.000

hCG: Human Chorionic Gonadotropin, PUL: Pregnancy of Unknown Location, OHSS: Ovarian Hyperstimulation Syndrome.

Discussion

In this trial, we observed shorter stimulation duration, lower gonadotropins consumption, and a lower number of retrieved oocytes in the GnRH antagonist group compared to the long agonist one.

Nevertheless, unlike stimulation duration, the effects on gonadotropins consumption and the number of retrieved oocytes were insignificant. However, based on our recent systematic review and meta-analysis on PCOS women [77], using conventional GnRH antagonist protocols led to a significantly lower gonadotropin consumption and a significantly lower number of retrieved oocytes compared to the long agonist one. (Gonadotropin consumption; WMD= -221.36, 95% CI: [-332.28 to -110.45] IUs; P < 0.0001; I^2 = 43%; χ^2-P = 0.1; high-quality evidence), (number of retrieved oocytes; WMD = 1.82, 95% CI: [-3.48 to -0.15] oocytes; P = 0.03; I2 = 58%; χ^2 P = 0.02; low-quality evidence), Thus, the insignificant result may relate to the relatively low sample size of the current trial. These effects may arise from the high levels of endogenous gonadotropins noted in the early follicular phase of conventional GnRH antagonist protocols, which reduce the total required dose of exogenous gonadotropins for stimulation. Moreover, higher LH levels promote FSH sensibility [78], while higher FSH leads to an uncoordinated and heterogeneous development of FSH-sensitive follicles [79, 80], which may, as a result, reduce the number of retrieved oocytes. In addition, the differences in the consumed dose of gonadotropins and the divergent effects of GnRH analogues on steroidogenesis may affect the regulation of ovarian angiogenesis. In the current study, we observed significantly lower FF PlGF levels in the GnRH antagonist protocol compared to the GnRH agonist one, which comes along with the result of Vrtačnik-Bokal et al. study [81] on PCOS subjects that reported lower FF VEGF levels in the GnRH antagonist group compared to the long GnRH agonist one. Gonadotropins regulate angiogenesis in GCs by promoting VEGF and hypoxia inducible factor-1α (HIF1α) expression and activity [82–86]. HIF1α is a transcription factor that acts as the master regulator of cellular response to hypoxia and serves as a critical mediator of the tissue's adaptive response to both low physiological oxygen levels and various pathological conditions [87] by regulating the oxygen-dependent transcription of many genes that control metabolism, angiogenesis, anti-apoptosis, and cell motility [88]. Since VEGF and PlGF genes have a hypoxia response element (HRE), HIF1α is capable of upregulating their transcription in hypoxic conditions [89, 90]. Moreover, FSH induces the expression of transforming growth factor-β (TGF-β) [91], which also controls VEGF expression and acts synergically with HIF-1α in upregulating VEGF expression [92]. Above that, Based on our recent systematic review and meta-analysis [77], treating PCOS women with the conventional GnRH antagonist protocols led to lower levels of estradiol on hCG day compared to the standard long agonist protocol (WMD= -259.21, 95% CI: [-485.81 to -32.60] pg/ml; P = 0.02; I^2 = 42%; χ^2-P = 0.10; moderate-quality evidence), which agrees with the previous in vitro studies that demonstrated lower aromatase activity and expression from granulosa lutein cells of GnRH antagonist-treated women compared to those of GnRH agonist-treated women [93]. Estrogen upregulates VEGF expression in ovarian [94] and endometrial cells [95, 96]. It also enhances the endometrial expression of PlGF and HIF-1α [96]. Thus, the lower dose of exogenous gonadotropins with the lower estrogen production in the GnRH antagonist protocol may create a lesser hypoxic environment compared to the long GnRH agonist one, which reduces the follicular fluid levels of the pro-angiogenic factors; VEGF and PlGF at the time of oocyte retrieval.

Several studies have reported that serum AMH levels decline during COH both in GnRH agonist and GnRH antagonist protocols [51, 97–100]. FF AMH levels are also lower in cycles stimulated with a GnRH

antagonist protocol compared to natural cycles [101–103]. The reduction of AMH levels during COH might be a result of the high dosage of exogenous FSH used during ovarian stimulation, which inhibits AMH production from the GCs, and allows more follicles to grow and maturate. However, previously, a significant positive correlation was noted between the decrease in AMH and the increase in estradiol plasma levels in FSH-stimulated cycles [104]. Based on the Grynberg et al. study, E2 has a stimulating effect on AMH through the estrogen-receptor-α (ER-α) and an inhibitory effect through estrogen-receptor-β (ER- β) [105]. Both ERs are expressed in the ovary, ERα is the predominant ER in thecal cells, and ERβ is the predominant ER in GCs until luteinization [56, 105, 106]. Thus, the effect of estradiol on AMH during the late follicular growth phase would therefore be mainly inhibitory [107]. However, it should be taken into account the dysregulation in the AMH/AMHR II controlling system in the GCs of PCOS subjects which may affect their response to COH. A recent study compared changes in AMH levels between low-ovarian reserve women, normo-ovarian reserve women, and PCOS women with hyperandrogenism during GnRH antagonist cycles showed a decline in AMH levels in both low-ovarian reserve and normo-ovarian reserve groups. In contrast, an initial increase in AMH levels preceding this decline was noted in PCOS women [97]. In addition, based on the Pierre et al. study, E2 down-regulated the expression of AMH and AMHR II in GCs of control women but did not affect these genes in GCs from PCOS subjects [57], which might be explained by the abnormal distribution of ER-α and ER-β in GCs of PCOS women [56–58] based on the data obtained from in-vitro studies on GCs. However, these studies disagreed on the nature of the abnormality; as one study noted an increase in ERα expression [57], while another showed a reduction in ER-β [58], and a third one reported a reduction in both receptors [56]. Thus, the type of GnRH protocol used in COH might have little or no effects on FF AMH levels in PCOS subjects, and further research is needed to clarify whether the effects are population-dependent and to determine the factors that mediate them in special populations like PCOS. However, if we assume that GnRH antagonist protocol is associated with a little higher FF AMH, together with the significantly reduced FF PlGF may explain the lower number of retrieved oocytes noted in this group. Nevertheless, the differences in the ovarian microenvironment did not affect the oocytes morphology or the pregnancy outcomes of IVF/ICSI. Thus, although GnRH antagonist protocol provides lesser stimulating effects on the ovary, it is sufficient enough to support pregnancy achievement. In this study, we focused on the hospitalized OHSS since it has the most adverse impact on the patient health and treatment cost. Interestingly, the rate of hospitalized OHSS did not differ significantly between the two groups, which might be a result of the elective embryo cryopreservation strategy that was followed, since in total more patients were susceptible to developing severe OHSS in the GnRH agonist group.

Strengths, limitations, and future research:

To the best of our knowledge, this is the first clinical trial that investigated the effect of GnRH agonist and GnRH antagonist protocols on the FF PlGF levels, FF AMH levels, and oocyte morphology in the PCOS population. However, our study has some limitations. First, due to the lack of drug supply during a certain point of the study duration, we used a non-random study design. Thus, our results may be affected by unknown bias or unmeasured confounding. Second, due to the limited budget, we only investigated the effects on the FF PlGF levels without measuring the levels of other VEGF family members or the

sVEGFR-1. Therefore, further research is needed to clarify the impacts on other angiogenesis regulators in PCOS and non-PCOS subjects. In addition, more data are required to understand the regulation process of PlGF expression in the female reproduction system since most of the available data were only concerned about VEGF.

Conclusions

Flexible GnRH antagonist protocol and the long GnRH agonist protocol regulate the follicular microenvironment and angiogenesis differently in PCOS subjects. However, these differences have no influence on the oocyte's morphology or the IVF/ICSI clinical outcomes. Thus, since flexible GnRH antagonist protocol represents a more patientfriendly and cost-effective protocol, it may be a better treatment choice for PCOS women undergoing IVF/ICSI.

Declarations

Data Availability:

The data that supports the findings are available upon request from the corresponding author.

Ethics declarations:

Competing interests: The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Author contributions:

All authors contributed to conceptualizing and designing the study. M.A. and S.K performed the clinical experiment and were responsible for the work in the field, including patients' recruitment, sample acquisition, and data collection. S.K. performed the statistical analysis and data interpretation. S.K. drafted the manuscript, while A.N. and M.A. revised it critically for important intellectual content. All authors approved the final manuscript.

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