Research Square

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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 normo-ovulatory women

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Research Article

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Abstract

Background

Gonadotropin-releasing hormone (GnRH) analogues are commonly used to prevent premature luteinizing hormone (LH) surge during In-Vitro Fertilization/ Intra-Cytoplasmic Sperm Injection (IVF/ICSI) cycles in routine practice. However, it is still unclear whether they have different effects on the follicular microenvironment in normo-ovulatory women.

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 83 normo-ovulatory women were allocated to take either the long GnRH agonist protocol (n = 50) or the flexible GnRH antagonist protocol (n = 33). 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 in any of the baseline characteristics. The FF PlGF levels were significantly lower, while FFAMH levels were insignificantly higher in the GnRH antagonist group (FF PlGF; GnRH agonist = 140.46 ± 42.46 pg/ml vs. GnRH antagonist = 120.49 ± 35.07 pg/ml; P value = 0.029). (FF AMH; GnRH agonist = 7.40 ± 5.69 ng/ml vs. GnRH antagonist = 8.51 ± 7.93 ng/ml; P value = 0.440). The stimulation duration was significantly shorter in the GnRH antagonist group compared to the long-agonist one (GnRH agonist = 8.28 ± 1.09 days vs. GnRH antagonist = 7.26 ± 0.89 days; P value < 0.001). 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 = 2523.00 ± 1034.11 IUs vs. GnRH antagonist = 2468.18 ± 879.53 IUs; P value = 0.952). However, there were not any significant differences between the two groups in embryological or clinical IVF/ICSI outcomes except for the endometrial thickness, which was thinner in the GnRH antagonist group, and the result almost reached

significance level (GnRH agonist = 9.66 ± 1.39 mm vs. GnRH antagonist = 9.03 ± 1.51 mm; P value = 0.058). In addition, the morphological assessment of MII oocytes showed comparable oocytes morphology in both groups.

Conclusions

Flexible GnRH antagonist protocol and the long GnRH agonist protocol regulate the follicular microenvironment and angiogenesis differently in normo-ovulatory women. However, these differences have no influence on the oocyte's morphology or the IVF/ICSI outcomes. In addition, although both protocols provide acceptable endometrial thickness, caution should be taken when designing the plans therapy for cases that are considered high risk of developing thin endometrial when stimulated with the flexible GnRH antagonist protocol.

Study registration:

This trial was prospectively registered at clinicaltrials.gov site under registration number (NCT04724343).

Introduction

Since the born of Louise Brown, the first In-Vitro Fertilization (IVF) baby, on 25 July 1978 [1], In-vitro Fertilization/ Intra-Cytoplasmic Sperm Injection (IVF/ICSI) techniques have widely been used for infertility management and facilitated the born of more than 8 million children [2]. Pituitary down-regulation is an essential step during controlled ovarian hyperstimulation (COH) to prevent premature luteinizing hormone (LH) surge [3], improve clinical pregnancy rate (CPR), reduce cycle cancellation rate (CCR) and increase the number of the retrieved oocyte [4, 5]. Gonadotropin-releasing hormone (GnRH) analogues are commonly used to accomplish pituitary down-regulation, but they differ in the mechanism of suppression. GnRH agonists initially cause a transient flare-up phase, followed by a down-regulation of GnRH receptors [3], while GnRH antagonists directly and rapidly inhibit gonadotropins secretion by competitively occupying the GnRH receptors [6]. Interestingly, GnRH receptors have also been identified in the ovary [7, 8], which suggest that, besides their central effects, GnRH analogues may have a direct impact on the ovary function.

PlGF is an angiogenic growth factor that belongs 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 [9]. It was initially isolated and cloned from a human placental cDNA library in 1991, from which it got the name [10], and it plays an important role in regulating placenta development and implantation [11–14]. Mice lacking the PlGF gene are healthy, fertile, without any obvious vascular impairment. However, they exhibit lower luteal angiogenesis during pregnancy compared to the wild type [15]. Moreover, they develop larger placentals, with thicker junctional zone (due to glycogen accumulation), thinner labyrinth layer, but comparable trophoblast morphology

and labyrinth vessel architecture compared to the control mice [16]. Besides, genetic deletion of PlGF resulted in smaller, less granular, and binucleated uterine natural killer cells compared to the wild type mice [17], which are known for their role in promoting arterial integrity, decidualization, trophoblast invasion, spiral arterial remodeling, early placental formation, and fetal growth [18]. However, recently, it has been reported that PlGF also regulates the ovarian function and ovulation [19]. PlGF is expressed in follicular fluids at higher levels compared to circulation, and its follicular fluid levels (FF PlGF) correlate positively with the number of retrieved oocytes of IVF/ICSI cycles [20]. However, it is still unknown whether the GnRH analogues may have different effects on the FF PlGF levels during COH.

Anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance (MIS), is a dimeric glycoprotein that belongs to the transforming growth factor (TGF)-β family. In male embryos, AMH facilities the regression of the Müllerian ducts, the Anlagen of the uterus, fallopian tubes, and upper vagina. While in females, it has an important role in regulation follicles growth and development [21–23] during both FSH-independent and FSH-dependent stages by inhibiting primordial follicles recruitment [24–26], attenuating the follicular responsiveness to FSH [27, 28], and inhibiting granulosa cell FSH-induced aromatase [28, 29]. AMH is produced mainly by granulosa cells (GCs) follicles in the ovaries, but the production level depends on the follicle development stage [30–32]. Based on the data of the in-vitro model, GnRH analogues may affect the expression of AMH from GCs differently [33, 34]. However, data from clinical trials on follicular fluid AMH (FF AMH) levels were inconsistent [35–38].

Typically, the oocyte quality is determined based on the nuclear maturation status and the presence of certain extra and intracytoplasmic morphologic features [39]. Although the impact of oocyte morphology on embryo competence and cycle outcomes is still controversial [40–45], evaluating the morphological characteristics of the oocytes after being denudated is considered a routine step during the ICSI process; in an attempt to improve the chance of obtaining and transferring embryos with a better implantation potential. During COH protocols, the supra-physiologic hormonal environment may have unfavorable effects on oocyte morphology as it induces the growth of a cohort of follicles, which, under natural conditions, would become atretic and regress [46]. However, it is still unclear whether these effects would depend on the protocol used with the inconsistent available data [39, 47–50].

Considering the previous data, we performed this study to evaluate the effects of the type of the GnRH analogues used in COH protocols on the follicular microenvironment of normo-ovulatory women and their reflections 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 normo-ovulatory women.

Materials And Methods

Study Design

This interventional, prospective, parallel, non-randomized, open-label controlled clinical trial was conducted on normo-ovulatory 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 NCT04724343.

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 83 normo-ovulatory women were allocated to take either the long GnRH agonist protocol (n=50) or the flexible GnRH antagonist protocol (n=33). Both the patients and the doctors were aware of the allocated arm. Patients who aged ≥ 40 years; or were diagnosed with polycystic ovary syndrome (PCOS), 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 [51]), 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 (Estradiol (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 [52].

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 ™ 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 ™ 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 G1-Plus ™ 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) [53]. 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 [54,55] or fulfill the criteria for OHSS hospitalization [56]. 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 inter-assay 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 [57]. 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 [57]. 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 [58]. 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 any of the baseline characteristics, as shown in Table 1. A significantly shorter stimulation duration was noted in the GnRH antagonist arm compared to the long-agonist one (GnRH agonist = 8.28 ± 1.09 days vs. GnRH antagonist = 7.26 ± 0.89 days; P value < 0.001; Table 2). On the other side, although the consumed dose of gonadotropins was lower in the GnRH antagonist group, the difference between the two groups was not statistically significant (GnRH agonist = 2523.00 ± 1034.11 IUs vs. GnRH antagonist = 2468.18 ± 879.53 IUs; P value = 0.952; Table 2). None of the participants developed OHSS required hospitalization, and no cycle was cancelled in any groups due to high risk of OHSS. Three cycles were cancelled in the GnRH agonist group due to fertilization failure. One of them was cancelled due to male factor (the male has cryptozoospermia and the female gave 23 oocytes, of which 12 were MII oocytes, 2 MI oocytes, 4 GV oocytes, and 5 atretic oocytes), while in the other two cycles, the women gave a relatively low number of MII oocytes. On the other hand, two cycles were cancelled in the GnRH antagonist group, of which one was cancelled due to thin endometrium, and the other due to male factor as no zygotes were obtained (the male has azoospermia and the female gave 27 oocytes, of which 20 were MII oocytes, 5 MI oocytes, and 2 atretic oocytes). However, there was no significant difference between the two groups in cycle cancellation rate. Similarly, the other cycle characteristics did not differ significantly between the two groups, as shown in Table 2. Interestingly, the FF PlGF levels were significantly lower in the GnRH antagonist group compared to the long-agonist one (GnRH agonist = 140.46 ± 42.46 pg/ml vs. GnRH antagonist = 120.49 ± 35.07 pg/ml; P value = 0.029; 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 = 7.40 ± 5.69 ng/ml vs. GnRH antagonist = 8.51 ± 7.93 ng/ml; P value = 0.440; Table 3). However, there were not any significant differences between the two groups in embryological or clinical IVF/ICSI outcomes (Table 3 and Table 4) except for the endometrial thickness, which was thinner in the GnRH antagonist group, and the result almost reached significance level (GnRH agonist = 9.66 ± 1.39 mm vs. GnRH antagonist = 9.03 ± 1.51 mm; P value = 0.058; Table 4). Moreover, the morphological assessment of MII oocytes showed comparable oocytes morphology in both groups (Table 3).

Table 1
Patients Baseline Characteristics.

	GnRH Agonist N = 50	GnRH Antagonist N = 33	P value
Female age (years)	28.12 ± 5.30	28.88 ± 6.29	0.555
Male age (years)	36.88 ± 7.13	37.58 ± 8.84	0.635
Infertility % (n):	74.0% (37/50)	78.8% (26/33)	0.618
Primary	26.0% (13/50)	21.2% (7/33)	
Secondary			
Infertility duration (years)	6.93 ± 4.06	5.54 ± 3.80	0.088
Smoker Male % (n)	44.0% (22/50)	63.6% (21/33)	0.080
Smoker Female % (n)	16.0% (8/50)	24.2% (8/33)	0.352
Male alcohol-consuming % (n)	0.0% (0/50)	6.1% (2/33)	0.155
Female alcohol-consuming % (n)	0.0% (0/50)	0.0% (0/33)	-
Male classification % (n):	10.0% (5/50)	27.3% (9/33)	0.208
Normozoospermia	24.0% (12/50)	21.2% (7/33)	
Mild-Moderate Male factor	46.0% (23/50)	24.2% (8/33)	
Oligoasthenoteratozoospermia	8.0% (4/50)	12.1% (4/33)	
Azoospermia	4.0% (2/50)	3.0% (1/33)	
Necrozoospermia	8.0% (4/50)	12.1% (4/33)	
Cryptozoospermia			

Table 2
Cycle Characteristics:

	GnRH Agonist N = 50	GnRH Antagonist N = 33	P value
Types of stimulator % (n):	64.0% (32/50)	51.5% (17/33)	0.410
r-FSH	8.0% (4/50)	6.1% (2/33)	
hMG	28.0% (14/50)	42.4% (14/33)	
r-FSH + hMG			
FSH starting dose (units)	294.00 ± 106.97	331.82 ± 107.76	0.104
Total FSH dose (units)	2523.00 ± 1034.11	2468.18 ± 879.53	0.952
Stimulation duration (days)	8.28 ± 1.09	7.26 ± 0.89	< 0.001
Sperms Source % (n):	70.0% (35/50)	81.8% (27/33)	0.384
Ejection	22.0% (11/50)	12.1% (4/33)	
Tesa	4.0% (2/50)	0.0% (0/33)	
Pesa	2.0% (1/50)	0.0% (0/33)	
Frozen	2.0% (1/50)	6.1% (233/)	
Ejection + Tesa			
Day of transfer	76.6% (36/47)	77.4% (24/31)	0.933
Day 2	23.4% (11/47)	22.6% (7/31)	
Day 3			
Cycle cancellation Rate % (n)	6.0% (3/50)	6.1% (2/33)	1.000
Cycle cancellation Rate due to risk of OHSS % (n)	0.0% (0/50)	0.0% (0/33)	-

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 = 50	GnRH Antagonist N = 33	P value
Number of Retrieved Oocytes	15.46 ± 6.05	16.24 ± 8.99	0.723
Ovarian Sensitivity Index	7.48 ± 4.75	7.37 ± 4.87	0.915
Number of Metaphase II Oocytes	9.08 ± 4.50	9.03 ± 5.34	0.595
Number of Metaphase I Oocytes	2.76 ± 1.57	3.18 ± 2.02	0.768
Number of GV Stage Oocytes	2.74 ± 2.31	3.33 ± 4.09	0.799
Number of Atretic Oocytes	0.88 ± 1.84	0.70 ± 1.67	0.814
Number of Fertilized Oocytes	5.88 ± 3.87	5.30 ± 3.50	0.556
Maturation Rate (%)	58.59 ± 21.12	56.81 ± 18.09	0.693
Fertilization Rate (%)	65.63 ± 28.41	59.75 ± 25.41	0.227
Number of Embryos Obtained	5.88 ± 3.87	5.30 ± 3.50	0.556
High-quality Embryos Rate (%)	60.26 ± 26.25	59.76 ± 26.23	0.790
Cleavage Rate (%)	94.00 ± 23.99	96.97 ± 17.41	0.539
Number of Embryos Transferred	4.32 ± 1.85	3.76 ± 1.90	0.155
FF AMH ng/ml	7.40 ± 5.69	8.51 ± 7.93	0.440
FF PlGF pg/ml	140.46 ± 42.46	120.49 ± 35.07	0.029
Oocytes Morphology % (n):	76.0% (38/50)	78.8% (26/33)	1.000
Normal	20.0% (10/50)	18.2% (6/33)	
Cytoplasmic Dysmorphisms	4.0% (2/50)	3.0% (1/33)	
Extra-Cytoplasmic Dysmorphisms	0.0% (0/50)	0.0% (0/33)	
Both			
Quantity of oocytes dysmorphisms % (n):	76.0% (38/50)	78.8% (26/33)	1.000
Normal	20.0% (10/50)	18.2% (6/33)	
One	4.0% (2/50)	3.0% (1/33)	
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 = 50	GnRH Antagonist N = 33	P value
Granulation % (n)	16% (8/50)	15.2% (5/33)	0.917
Refractile Bodies % (n)	0.0% (0/50)	0.0% (0/33)	-
SER % (n)	0.0% (0/50)	0.0% (0/33)	-
Vacuoles % (n)	6.0% (3/50)	3.0% (1/33)	1.000
Dark Cytoplasm % (n)	2.0% (1/50)	3.0% (1/33)	1.000
Oocytes Shape % (n)	0.0% (0/50)	0.0% (0/33)	-
Oocytes Size % (n)	0.0% (0/50)	0.0% (0/33)	-
ZP Dysmorphisms % (n)	0.0% (0/50)	0.0% (0/33)	-
PVS Dysmorphisms % (n)	0.0% (0/50)	0.0% (0/33)	-
PB Dysmorphisms % (n) (Duplicated/ Triplicated PB)	4.0% (2/50)	3.0% (1/33)	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 = 50	GnRH Antagonist N = 33	P value
Endometrial thickness on hCG day (mm)	9.66 ± 1.39	9.03 ± 1.51	0.058
Biochemical Pregnancy Rate % (n)	36.0% (18/50)	30.3% (10/33)	0.591
Per Woman:	38.3% (18/47)	32.3% (10/31)	0.586
Per Embryo Transfer:			
Clinical Pregnancy Rate % (n)	30.0% (15/50)	27.3% (9/33)	0.789
Per Woman:	31.9% (15/47)	29.0% (9/31)	0.787
Per Embryo Transfer:			
Ongoing Pregnancy Rate % (n)	24.0% (12/50)	24.2% (8/33)	0.980
Per Woman:	25.5% (12/47)	25.8% (8/31)	0.978
Per Embryo Transfer:			
Multiple Pregnancy Rate % (n)	14.0% (7/50)	12.1% (4/33)	1.000
Per Woman:	14.9% (7/47)	12.9% (4/31)	1.000
Per Embryo Transfer:			
Implantation Rate %	9.43 ± 16.02	10.81 ± 20.59	0.940
Resolved PUL Rate% (n)	4.0% (2/50)	3.0% (1/33)	1.000
Per Woman:	4.3% (2/47)	3.2% (1/31)	1.000
Per Embryo Transfer:			
Ectopic Pregnancy Rate % (n)	0.0% (0/50)	0.0% (0/33)	-
Per Woman:	0.0% (0/47)	0.0% (0/31)	
Per Embryo Transfer:			
Hospitalized OHSS Rate % (n)	0.0% (0/50)	0.0% (0/33)	-

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

Discussion

Based on previous in vitro studies, treating GCs with a GnRH antagonist decreases their expression of AMH compared to the GnRH agonist [33, 34]. However, it was unclear what stimulating conditions those

GCs had undergone before being involved in the in vitro experiments. In our study, insignificantly higher FF AMH levels were noted in the GnRH antagonist group compared to the long agonist one. Serum [59–62] and FF AMH levels [21, 63, 64] are reduced during COH cycles, which can be explained by the high dose of exogenous FSH used in COH protocols that inhibits AMH production from the granulosa cells, and allows more follicles to develop and maturate. However, in FSH-stimulated cycles, this decrease in AMH levels is positively correlated with the increase of plasma E2 levels [65]. E2 can both stimulate and inhibit AMH expression through estrogen-receptor-α (ER-α) and estrogen-receptor-β (ER-β), respectively [66]. Both ERs are expressed in the ovarian cells, ERα is the predominant ER in thecal cells, and ERβ is the predominant ER in GCs until luteinization [66–68]. Therefore, during the late follicular growth phase, E2 effects would be generally inhibitory [69]. However, since GnRH antagonist protocols are associated with lower gonadotropins consumption, aromatase activity, and E2 production [33, 70, 71], it makes sense for FF AMH levels to be higher in the GnRH antagonist group. Nevertheless, in our study, the effects did not reach significance levels, which comes along with the results of Kaya et al. [36] and Lee et al. (2010) [37] that also noted insignificant higher FF AMH levels in the GnRH antagonist arm. However, the differences between the two protocols were significant in the Ilhan et al. study [38]. On the other hand, the earliest study of Lee et al. (2008) noted insignificant lower FF AMH in the GnRH antagonist group despite the significantly lower gonadotropins dose and lower serum E2 on hCG day in this group. Thus, the type of GnRH protocol used might have little or no effects on FF AMH levels, and these effects may be influenced by the type of study population. Although the women were normoovulatory with either tubal or male infertility factors in our trial, the other studies involved a much general IVF/ICSI population by including unexplained infertility, male factor infertility, tubal factor infertility, endometriosis (with excluding endometriosis stage III-IV), and anovulatory women. In addition, Ilhan et al.'s study [38], in which the results showed significant differences, included women with decreased ovarian reserve.

On the other side, we observed significantly lower FF PlGF levels in the GnRH antagonist group compared to the GnRH agonist one. Although no previous study has investigated the impact of GnRH analogues on FF PlGF levels, two studies, with inconsistent results, compared the effects of the flexible GnRH antagonist protocol and the long agonist one on the FF VEGF levels. Ferrari et al.'s study [72] showed higher FF VEGF levels in the GnRH antagonist group, while the differences between the two protocols were insignificant in the Kaya et al. study [36]. We should take into account that although the women of the study of Ferrari et al. [72] were not poor-responders, they might be considered as hypo-responders since they required a higher-than-expected dose of the stimulator, especially in the GnRH agonist group, in which the mean of consumed dose ± SD was (3546.7 ± 1842.53 IUs) while the mean ± SD of retrieved oocytes was only (7.03 ± 3.36 oocytes), which might have disturbed the FSH effects on ovarian folliculogenesis and angiogenesis. In addition, Ferrari et al. [72] included endometriotic women, while Kaya et al. study [36] only excluded endometriosis stage III-IV, and FF VEGF is dysregulated in this study population [73, 74]. Therefore, differences in patients' characteristics should also be considered. Several factors are contributed to the regulation of VEGF and PlGF expression in the female reproductive system. Some reports showed that gonadotropins might control GCs angiogenesis by enhancing VEGF and hypoxia inducible factor-1α (HIF1α) expression and activity [75–79]. HIF1α is a transcription factor that

mediates the cellular adaptive response to hypoxia [80], and in hypoxic conditions, it upregulates the transcription of VEGF and PlGF genes since they have a hypoxia response element (HRE) [81, 82]. Besides, FSH enhances the expression of transforming growth factor-β (TGF-β) [83], which plays a synergistic role with HIF-1α in upregulating VEGF expression [84]. On the other hand, Estrogen enhances the endometrial expression of PlGF, HIF-1α [85], and VEGF [85, 86]. Thus, since the GnRH antagonist protocol is considered a less intensive protocol (with requiring a lower dosage of gonadotropins), in addition to its effect in lowering E2 production, it may promote ovarian angiogenesis during COH to a lesser extent compared to the long GnRH agonist one. However, although this did not adversely affect ovarian function, as no significant differences were noted in any embryological IVF/ICSI outcomes between the two groups, it might contribute to the thinner endometrial thickness observed in the GnRH antagonist group if it has similar impacts on the endometrial angiogenesis. Nevertheless, the final implantation rate and pregnancy rates were comparable between the two protocols. Data regarding GnRH antagonist impact on endometrial receptivity are conflicted as some clinical studies showed that the endometrial receptivity of GnRH antagonist cycles was more similar to the natural cycle receptivity than that of GnRH agonist cycles [87, 88], while others revealed harmful effects [89–92] that may be mediated by downregulation the endometrial expression of some endometrial receptivity markers like HOXA-10 [89, 90], leukemia inhibitory factor (LIF), and integrin β3 subunit [92]. In addition, GnRH upregulates endometrial Allograft inflammatory factor-1 (AIF-1) expression, a cytokine associated with inflammation and allograft rejection, which might be unfavorable for embryo implantation as increased AIF-1 might inhibit adhesion during implantation via raised Tumor necrosis factor-α (TNF-α) [91]. In addition, although the higher levels of endogenous gonadotropins noted in the early follicular phase of conventional GnRH antagonist protocols may shorten the stimulation duration and lower the total required dose of exogenous gonadotropins for stimulation, higher exposure of the genital tract to LH and E2 at this early phase might adversely affect the implantation rate mainly by altering endometrial receptivity [93]. In routine practice, CPR and LBR decrease for each millimeter of endometrial thickness declines below 8 mm in fresh IVF cycles [94]. In our trial, two women (4.0%) in the long GnRH agonist arm and four women (12.12%) in the GnRH antagonist one had endometrial thickness lower than 8 mm, but except for the cycle cancellation case, none of them had endometrial thickness lower than 7 mm. Thus, in general, the endometrial thickness in both groups was relatively acceptable, which was confirmed with the comparable pregnancy rates. However, caution should be taken when designing the plans therapy for cases that are considered high risk of developing thin endometrial like Asherman syndrome, history of uterine surgery, infection, or radiation [95].

Regarding oocytes dysmorphisms, the results of our study come along with the results of Cota et al. randomized controlled trial [48], which showed comparable oocytes morphology in the GnRH antagonist protocol and the long GnRH agonist protocol. We also agreed with the result of the retrospective study of Rienzi et al. [49], in which the author compared the effect of three different controlled ovarian stimulation protocols; GnRH agonist Long protocol (268 cycles), GnRH antagonist protocol (142 cycles), and natural cycle with minimal stimulation (106 cycles), but could not detect any correlation between oocyte morphology, and the type of protocol used [49]. However, our results disagreed with other study results

[39, 47, 50]. The studies of Ebner et al. [47] and Murber et al. [50] have reported worse oocyte morphology in the GnRH antagonist group, with lower zona scores [47] and higher rates of cytoplasmic dysmorphisms [50]. Differently, Zanetti et al. [39] showed lower incidences of dark cytoplasm, smooth endoplasmic reticulum cluster (SER), and abnormality in zona pellucida in the GnRH antagonist group. The inconsistency in results may arise from the differences in study design, as except for Ebner et al. [47] and Cota et al. [48] studies, all the other reports were retrospective studies. In addition, these studies used different types of gonadotropin stimulators. Ebner et al. [47] used two different gonadotropin stimulators in the studied groups; with human menopausal gonadotrophin (hMG) in the agonist group and recombinant follicle-stimulating hormone (r-FSH) in the antagonist one, which may explain the superiority that the authors noted in the GnRH agonist group, as some reports suggested the using hMG may improve the oocyte morphology [96, 97]. Above that, Although the other studies used similar types of gonadotropins for both groups (Rienzi et al. [49] and Zanetti et al. [39] studies used r-FSH, while Murber et al. study [50] matched the type of gonadotropin using hMG or high-purified urofollitropin between the two groups), they did not provide sufficient information about the study population' characteristic. Thus, differences in study populations can't be excluded. Previous reports suggested that some diseases, e.g., endometriosis [98–101], and even some social habits, e.g., smoking [102], may have detrimental effects on oocyte morphology and quality. In our studies, there were no significant differences between the two groups in any patient's characteristics. In addition, we excluded all currently known possible confounders.

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 in normo-ovulatory women. However, our study has some limitations. First, due to the lack of drug supply during a certain point of the study duration, we changed the study design into a non-randomized controlled trial. 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 sVEGFR1. Therefore, further research is needed to clarify the impacts on other angiogenesis regulators. In addition, more studies are required to confirm whether the GnRH antagonist effect on FF PlGF levels would be reproducible on other tissue like the endometrium and placenta.

Conclusions

Flexible GnRH antagonist protocol and the long GnRH agonist protocol regulate the follicular microenvironment and angiogenesis differently in normo-ovulatory women. However, these differences have no influence on the oocyte's morphology or the IVF/ICSI outcomes. In addition, although both protocols provide acceptable endometrial thickness, caution should be taken when designing the plans therapy for cases that are considered high risk of developing thin endometrial when stimulated with the flexible GnRH antagonist protocol.

Abbreviations

- AIF-1: Allograft inflammatory factor-1.
- AMH: Anti-Müllerian Hormone.
- BPR: Biochemical Pregnancy Rate.
- CCR: Cycle Cancellation Rate.
- COCs: Cumulus Oocyte Complex.
- COH: Controlled Ovarian Hyperstimulation.
- CPR: Clinical Pregnancy Rate.
- E2: Estradiol.
- ER: Estrogen Receptor.
- FF: Follicular Fluid.
- GCs: Granulosa Cells.
- GnRH: Gonadotropin-Releasing Hormone.
- GV oocytes: Germinal Vesicle Oocytes.
- hCG: human Chorionic Gonadotropin.
- HIF-1α: Hypoxia Inducible Factor-1α.
- hMG: human Menopausal Gonadotropin.
- HRE: Hypoxia Response Element.
- ICSI: Intra-Cytoplasmic Sperm Injection.
- IR: Implantation Rate.
- IVF: In-vitro Fertilization.
- LH: Luteinizing Hormone.
- LIF: leukemia inhibitory factor.
- MI oocytes: Metaphase I Oocytes.
- MII oocytes: Metaphase II Oocytes.
- MIS: Müllerian Inhibiting Substance.
- MPR: Multiple Pregnancy Rate.
- OHSS: Ovarian hyperstimulation syndrome.
- OPR: Ongoing Pregnancy Rate.
- OSI: Ovarian Sensitivity Index.
- PB: Polar Body.
- PCOS: Polycystic Ovary Syndrome.
- Pesa: Percutaneous Epididymal Sperm Aspiration.
- PlGF: Placental Growth Factor.

- PVS: Perivitelline Space.
- r-FSH: recombinant Follicle-Stimulating Hormone.
- RPUL: Resolved Pregnancy of Unknown Location.
- SC: Subcutaneously.
- SER: Smooth Endoplasmic Reticulum Aggregations.
- sVEGFR-1: Soluble form of VEGF receptor-1.
- Tesa: Testicular Sperm Aspiration.
- TGF-β: Transforming Growth Factor-β.
- TNF-α: Tumor necrosis factor-α.
- VEGF: Vascular Endothelial Growth Factor.
- ZP: Zona Pellucida.

Declarations

Ethics approval and consent to participate:

The Ethical Committee of Damascus University approved the study protocol, and a written informed consent was obtained from all participants.

Consent for publication:

Not applicable.

Availability of data and materials:

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

Competing interests:

The authors declare that they have no competing interests.

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Authors' 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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