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The effect of dual stimulation on ploidy rates in patients with poor ovarian response

Elif Ganime Aygün, Emine Karabük

Department of Obstetrics and Gynecology, Acibadem Mehmet Ali Aydinlar University Atakent Hospital, Istanbul, Turkey

ORCID ID of the author(s)
EGA: 0000-0003-3737-7250
EK: 0000-0003-2055-3000

Corresponding Author
Elif Ganime Aygün
Department of Obstetrics and Gynecology, Acibadem Mehmet Ali Aydinlar University Atakent Hospital, Turgut Özal Bulvarı no:16 Küçükçekmece, İstanbul, Turkey
E-mail: gynelifaygun@gmail.com

Ethics Committee Approval
Ethics committee approval for the study was obtained from the Ethics Committee of Acıbadem Mehmet Ali Aydınlar University (ATADEK) with the decision number 2021-01/14.
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: It is difficult to obtain healthy oocytes in poor ovarian response patients with conventional treatment methods. This study aimed to find out which agent is most effective at inducing ovulation in one menstrual cyle. We aimed to examine the effects of follicular and luteal stimulation in patients with poor ovarian response on oocyte count, blastocyst, euploid embryo, and pregnancy rates.
Methods: A total of 134 patients were included in this retrospective cohort study, and the rates of ploidy in the embryos obtained by follicular and luteal stimulation were evaluated. All cases were treated with the antagonist protocol beforehand. The research was conducted by examining the data of patients who underwent Dual stimulation (Duostim) between 2015 and 2017 in the IVF Clinic of Acıbadem University Atakent Hospital retrospectively.
Results: While ploidy rates in FS and LS were significant in terms of age and AMH values ($P<0.05$ for all), they did not differ with BMI values ($P>0.05$ for all). The rate of aneuploid embryo development in follicular phase was 81.8%, while that in luteal phase was 18.2%, and the rates of euploid embryo development in follicular and luteal treatments were 34.6% and 18.2%, respectively. The pregnancy rate with euploid embryos by LPS was significantly higher compared that by FPS ($P<0.05$).
Conclusion: Our study concludes that follicles entering the anovulatory phase in the follicular phase can be saved by LS, so that healthier embryos can be obtained.

Keywords: Duostim, Poor-responder patients, Double ovarian stimulation, Reduce ovarian reserve

Introduction

The worldwide prevalence of infertility ranges between 2.5-10.5% in women [1]. Some of these women must undergo assisted reproductive techniques, and the poor prognosis group consists of those with advanced age and poor ovarian response. The rate of poor ovarian response ranges between 5.6- 35.1% among infertile women. It may be necessary to implement a special treatment protocol in these patients to obtain high-quality oocytes and increase the pregnancy rate [2, 3]. Although there is still no clear protocol, transferring the embryos with a freeze-thaw protocol to use a hormonally unstimulated endometrium may be beneficial in increasing pregnancy rates [4, 5].

A treatment plan, known as the POSEIDON group (Patient-Oriented Strategies Encompassing IndividualizeD Oocyte Number), is suggested to determine the correct oocyte quantity and sufficiency [6]. It was concluded that a healthy pregnancy can be achieved after finding a healthy embryo by a good blastocyst culture and euploidy study [7, 8].

The most difficult patient group in assisted reproductive techniques is those with weak ovarian response. Numerous and high-quality embryos cannot be obtained in these patients with standard ovarian stimulations. Luteal phase stimulants and embryo freezing technology come into play at this point [9].

For this reason, follicular and luteal phase stimulants (dual stimulation, duostim) administered in the same menstrual cycle in addition to luteal phase stimulation are used in the treatment of patients with weak ovarian capacity, especially in cancer patients who want to urgently preserve their reproductive function [10, 11].

Duostim protocols are less preferred than traditional treatments due to time consumption and high cost. Cycle cancellation is also more frequent with dual stimulation [12, 13].

For patients undergoing Duostim, ESHRE identified patients with poor ovarian response using the Bologna criteria [14]. Accordingly, at least two of the following three criteria must be met:

1. Advanced maternal age (40 years or older) and any other risk factors for poor ovarian response
2. Obtaining 3 or fewer oocytes with a previously conventional stimulation protocol
3. Abnormal ovarian reserve test (AFC<5-7 or AMH<0.5-1.1 ng/ml)

Before these criteria, different definitions of weak ovarian response were made [12, 15, 16].

Compared to conventional treatment, dual stimulation is somewhat superior to the traditional method in terms of the number of eggs, mature eggs, and blastocysts obtained. After two phases of stimulation, higher oocytes and embryos were obtained in the luteal phase [17-23]. Additionally, the number of normal karyotype blastocysts were higher [24].

Choosing a personalized treatment that will increase success and reduce complications is the most significant measure in ovarian stimulation. Selection of the right stimulation agent, the use of agonists or antagonists to reduce LH (luteal hormone) peak, hCG or agonist trigger use to obtain a mature oocyte, fresh or frozen embryo transfer, and whether the embryo is selected with pregenetic diagnosis or morphological characteristics are the key steps. The main goal in all is to keep the ovarian response at a maximum in patients with a weak response [25].

There may be more than one follicular wave in a human ovarian cycle, which eradicates conventional stimulation protocols [26].

Dual stimulation, that is, duostim follicular phase stimulation (FPS) that complements luteal phase stimulation (LPS), is implemented to preserve fertility in patients with a low ovarian reserve and advanced age [19, 27]. In addition, duostim can be used in all patients to increase the number of mature oocytes and increase the cumulative birth rate. It also shortens the time to obtain euploid blastocyst [28, 29].

Cimadomo et al. [30] compared follicular and luteal phase stimulations in the same cycle to evaluate the efficacy of treatment.

In this article, we aimed to examine the effects of follicular and luteal stimulation in patients with poor ovarian response on oocyte count, blastocyst, euploid embryo, and pregnancy rates. We intended to provide an unbiased perspective on the number of oocytes and healthy blastocysts obtained after the treatment by evaluating the number of healthy embryos after two stimulations given in one cycle.

Materials and methods

This retrospective cohort study evaluated the data of patients who underwent Dual stimulation (Duostim) between 2015 and 2017 in the IVF Clinic of Acıbadem University Atakent Hospital. Ethics committee approval for the study was obtained from the Ethics Committee of Acıbadem Mehmet Ali Aydınlar University (ATADEK) with the decision number 2021-01/14. There were 134 patients in the study, and the rates of ploidy in the embryos obtained by stimulatory and luteal stimulation were evaluated. The patients consisted of women who had previously used an antagonist protocol due to poor ovarian response, from whom insufficient oocytes were collected, or blastocysts or a genetically healthy embryo could not be obtained.

Inclusion criteria

Being under the age of 41 years, having a menstrual cycle length between 21 and 35 days, having an indication for starting treatment with at least 300 IU, having both ovaries in place and not having undergone ovarian surgery, having received antagonist protocol treatment with failure to obtain a euploid embryo, FSH level not exceeding 15 IU/ml and LH level not exceeding 12 IU/ml.

Exclusion criteria

Presence of follicles larger than 10 mm before treatment, endometriosis stage 3 or 4, and concomitant uterine pathology (adenomyosis, submucous myoma, Asherman syndrome), and having an azoospermic partner.

Stimulation protocol

Dual stimulation was performed to all patients who were treated. After screening and baseline evaluation of the ovaries at gynecological examination, fixed-dose recombinant FSH (rec-FSH) (300 IU/day; Gonal-F, Merck-Serono, Germany; Puregon, MSD, USA) was administered for 4 days. Follicular growth was monitored on day 5 and then every 2 days. A gonadotropin releasing hormone antagonist (GnRH antagonist) (Cetrorelix, Cetrotide, Merck-Serono; Ganirelix, Orgalutran, MSD) was administered daily (in a single subcutaneous dose)

after identification of a prominent follicle ≥13-14 mm in diameter and until the day of the ovulation trigger. A bolus dose of 0.5 ml buserelin (Suprefact, Hoechst Marion Roussel, Germany) was administered when at least two follicles reached a diameter of ≥17-18 mm. Oocyte retrieval was performed 35 hours after the trigger. Approximately 5 days after the first oocyte retrieval, that is, when complete luteinization was achieved, recombinant LH (rec-LH) (300 IU/day; Luveris, Merck- Serono) was started with the same protocol and daily dose as for FPS, regardless of the number of antral follicles counted in the scan. Oocyte collection was performed with the same pick-up protocol. After oocyte collection, intracytoplasmic sperm injection was performed, and an embryo culture medium was created. Oocytes were collected from the follicles by transvaginal ultrasound-guided aspiration and cultured for 2-3 hours in a culture medium (CSCM, Irvine Scientific, Australia) at 37°C, 5% CO_2, and 5% O_2. Then, peeling and fertilization were performed in a Hepes-buffered medium (Irvine Scientific). Fertilization was assessed 16-20 hours after intracytoplasmic sperm injection by the presence of two equally sized pronuclei. Embryo culture was performed in a single 25 µl microdrop CSCM in a benchtop incubator (MINC, Cook Medical, USA) in a controlled humidified atmosphere until the fully expanded blastocyst stage (Day 5).

After laser-assisted zona dehiscence, a trophectoderm biopsy was performed. Biopsies were obtained from all embryos that developed as viable blastocysts, regardless of their morphological quality and/or full expansion days. After trophectoderm biopsy, collapsed blastocysts were vitrified with Cryotop devices and solutions (Kitazato BioPharma Co., Japan).

Embryo vitrification protocol

Vitrification was performed using the Cryotop device and solutions (Kitazato BioPharma Co., Japan). Initial equilibration was carried out in 7.5% ethylene glycol and 7.5% dimethyl sulfoxide at room temperature for 12-15 minutes. Embryos were then transferred for 1 minute into 15% ethylene glycol, 15% dimethyl-sulfoxide, and 0.5 M sucrose, then placed on the Cryotop film strip as a single small drop. Excess solution was removed leaving only a thin layer around each embryo and the Cryotop was submerged in liquid nitrogen. The strip was capped, and the sample was stored by immersion in liquid nitrogen.

Embryo thawing protocol

On warming, the Cryotops were removed from liquid nitrogen and the Cryotop's filmstrip was rapidly immersed in 1 ml of 37°C warming solution containing 1.0 M sucrose for 1 minute, then the oocytes and embryos were transferred to a room temperature solution containing 0.5 M sucrose and incubated for 3 minutes. After two consecutive washings in a basic medium at room temperature for 6 minutes each, the embryos were placed in a 1 ml culture medium (Cleavage medium, Sage).

Pregenetic diagnostic analysis

Trophectoderm biopsy was performed on the 5th embryos [31]. All biopsy procedures were performed in 10 ml of HEPES buffered medium (Quinn's Advantage, Cooper Surgical) coated with pre-equilibrated mineral oil. The laser was used to help drill a 10–20-micron hole into the embryo's outer wall. 5-10 trophectoderm cells were then aspirated into a trophectoderm biopsy pipette (research instrument), and the cells were removed from the embryo body with the help of the laser. All embryos were frozen by vitrification after the biopsy and sent to the genetics laboratory for chromosome analysis.

Embryo transfer

After the detection of euploid embryos and spontaneous follicle development, which began on the 7th day of the cycle, the patients were called to the outpatient clinic every 2 days for vaginal ultrasonography and LH measurements. Embryo transfers were performed with the natural cycle transfer on the 7th day of ovulation. Dydrogesterone 10 mg was administered 3 times a day for luteal support.

Statistical analysis

Descriptive statistics were used to define continuous variables (mean, standard deviation, minimum, median, maximum). Frequencies (n) and percentages (%) were used to define the categorical variables. Independent and non-normally distributed continuous variables were compared with the Mann-Whitney U test, and two independent and normally distributed continuous variables were compared with the student's t-test. Chi-Square (or Fisher Exact test, where appropriate) was used to examine the relationship between the categorical variables.

The statistical significance level was set at 0.05 and SPSS 24.0 program was used for all statistical analyses.

Results

One hundred and thirty-four patients who had previously failed treatment with the antagonist protocol were evaluated retrospectively. In the FPS and LPS stimulation steps of the dual stimulation performed in the same cycle, AMH, BMI, age-related ploidy ratios, in addition to the ploidy rates of the two treatments were comparatively evaluated (Tables 1 and 2).

Table 1: Comparisons according to FSP

	FSP 0 (n=96)		FSP 1 (n=37)		
	Mean (SD)	Med. (Min-Max)	Mean (SD)	Med. (Min-Max)	*P*-value
Age	36(4)	38 (26-40)	34(4)	35 (27-40)	0.001
AMH	0.98(0.58)	0.98 (0.01-2.34)	1.27(0.5)	1.23 (0.14-2.4)	0.004
BMI	26(4)	26 (19-37)	28(4)	28 (20-33)	0.058
FSH blood level	9.04(2.25)	9 (5-14)	7.97(1.98)	8 (5-13)	0.008
LH blood level	9.35(1.82)	9 (6-13)	8.57(1.52)	8 (6-12)	0.025
Afc	5.64(2.19)	6 (1-10)	6.81(1.65)	7 (4-12)	0.009
E2 blood level	31.3(8.64)	31.5 (17-54)	33.1(7.65)	34 (17-49)	0.232
FSP oocyte count	1.82(1.2)	2 (0-6)	2.38(1.01)	2 (0-5)	0.011
LPS oocyte count	2.64(1.44)	2 (0-7)	3.11(1.02)	3 (1-5)	0.024

Mann-Whitney U test, FSP: Follicular stimulation protocol, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, Afc: Antral Follicle Count, E2: Estradiol

Table 2: Comparisons according to LSP

	LSP 0 (n=55)		LSP 1 (n=78)		
	Mean (SD)	Med. (Min-Max)	Mean (SD)	Med. (Min-Max)	*P*-value
Age	36(4)	38 (26-40)	35(4)	36 (27-40)	0.017
AMH	0.91(0.55)	0.9 (0.01-2.34)	1.17(0.57)	1.2 (0.1-2.4)	0.003
BMI	26(4)	27 (19-35)	27(4)	27 (19-37)	0.847
FSH blood level	9.18(2.18)	9 (5-14)	8.44(2.21)	8 (5-14)	0.026
LH blood level	9.49(1.73)	9 (7-13)	8.88(1.77)	9 (6-13)	0.071
Afc	5.64(2.34)	6 (1-10)	6.19(1.91)	6 (2-12)	0.264
E2 blood level	30.6(9)	31 (17-49)	32.63(7.87)	33 (18-54)	0.117
FSP oocyte count	1.84(1.23)	2 (0-6)	2.08(1.13)	2 (0-5)	0.283
LSP oocyte count	2.56(1.34)	2 (0-6)	2.91(1.34)	3 (1-7)	0.165

Mann-Whitney U test, FSP: Follicular stimulation protocol, LSP: Luteal stimulation protocol, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, Afc: Antral Follicle Count, E2: Estradiol

While ploidy rates in FPS and LPS were significant in terms of age and AMH values (P<0.05), they did not differ with BMI values. However, the rate of aneuploid embryo development in FPS was 81.8% while that in LPS was 18.2%, and the rates of euploid embryo development in FPS and LPS treatments were 34.6% and 18.2%, respectively. The pregnancy

rate with euploid embryos by LPS was significantly higher compared to that by FPS (P<0.05) (Table 3).

Table 3: The comparisons according to LSP and FSP

		LSP				
		0		1		
		n	%	n	%	*P*-value
FSP	0	45	81.8	51	65.4	0.049
	1	10	18.2	27	34.6	

Fisher's Exact test, FSP: Follicular stimulation protocol, LSP: Luteal stimulation protocol

The main goal was to compare the rates of euploid embryos after FPS and LPS. To find the cut-offs of age, AMH, FSH blood level, LH blood level, AFC, E2 (estradiol) blood level, FSP oocyte number, LPS and oocyte number, ROC analysis was performed with FSP=0, and LSP=1, which revealed no significant differences in the areas under the curve (AUC) (P>0.05). The cut-offs were uninterpretable.

Discussion

Dual stimulation aims to obtain a higher number of oocytes compared to FPS. However, the main reason may also be the synchronized follicular development due to hormonal levels in the LPS period [30]. This study aimed to show the specific step of dual stimulation in which more euploid embryos can be obtained in patients with poor ovarian response and to determine whether the euploid embryo ratio is affected by AMH, BMI, and age. All our patients had a weak response, were previously treated with the antagonist protocol, and they could not get pregnant. According to the poor ovarian criteria, the baby birth rate increases in women with controlled ovarian stimulation when stimulation is performed at most 3 times. However, these patients usually drop the process because they are tired of the treatment [34].

While the unsuccessful result is the first reason for discontinuation of treatment, the second reason is financial factors [35, 36]. In dual stimulation, using a double stimulation agent in one cycle and obtaining more embryos prevents the abandonment of the process and increases the chance of obtaining euploid embryos. Only 9% of patients continue controlled ovarian stimulation, which is low [36]. In our clinic, patients receive two-cycle treatments in dual stimulation although they pay the single cycle cost. We perform dual stimulation in patients who meet the Bologna criteria and had failed controlled ovarian stimulation, and a ploidy scan to achieve a successful pregnancy.

Embryo quality was better in luteal stimulation than in follicular phase stimulation in the previous studies, as in ours [17-19, 22]. However, the live birth rates between luteal and follicular phase stimulation protocols were similar in studies with large series [37, 38].

In LPS, the reason for a good quality oocyte is a possible exacerbation because of the GnRH agonist stimulation used in FPS. This anovulatory wave creates a downregulation of AMH expression and increases the number of follicles with a diameter of 3-4 mm in LPS. Of course, the effect of endocrine and paracrine factors should also be confirmed, because, in some studies of Luo et al. [39], there was no significant difference between embryo quality and ploidy rates of patients given FPS and LPS.

High levels of estrogen and progesterone during the luteal phase may induce a more synchronous follicular development and promote FSH receptors in the granulosa cells [30].

The possibility of obtaining a euploid blastocyst in either of the two phases of the ovarian cycle suggests that non-dominant follicles may become more prominent and develop randomly. In other words, the dominant follicle is not a competent follicle for a good embryo. This perspective may enable us to focus on wave theories in follicle development and provide a different understanding of ovarian physiology. Such an interesting topic could also trigger future studies.

To evaluate the ovarian, clinical, and even postnatal outcomes of LS, not only patients with a poor ovarian response but rather wider case series should be investigated. In this regard, Chen et al. [38] compared LS with the traditional method in 2015 and reported that there was no difference in terms of birth data and congenital anomalies. However, this study was also performed retrospectively.

LH selection in dual stimulation is intended to support steroidogenesis and folliculogenesis [40, 41]. LH increases androgen production and the stimulation of preantral/antral follicles as well as FSH receptor expression in granulosa cells [42]. All these features are important in patients with advanced maternal age, decreased androgen sensitivity due to age, and endogenously deficient androgen. In this group of patients, the ovarian response to exogenous FSH is also extremely weak [43]. Although a therapeutic LH dose is not recommended in antagonist protocols involving controlled ovarian stimulation, adding LH to the treatment in patients with poor response achieves a higher chance of success with low r-FSH doses [44]. Despite all this, dual stimulation is still debated by the scientific community.

The development of follicles was monitored in many animal models before being studied in women. Dual stimulation is deemed suitable for the treatment of patients with a poor response in terms of both ovulatory and anovulatory fluctuations. It is mostly recommended for patients who need to preserve fertility for medical reasons. The advantage of dual stimulation is obtaining more eggs and embryos in a single cycle. Especially in LS, higher blastocyst and euploidy rates compared to those obtained in FS supported the use of the dual protocol in patients who will experience ovarian failure due to medical reasons and who have time constraints [25]. However, more research is needed on this subject.

Limitation

For more generalizable results, larger study groups and more parameters are needed. However, we aimed to determine the stage of treatment at which a euploid embryo can be obtained. Perhaps the same rates of euploidy could be achieved in randomized sequential controlled hyperstimulation. The retrospective design was another limitation, and the duostim protocol should have had a matched control group. Another limitation is that it has already been proven that the success rate is higher in frozen embryos [32, 33]. More data on luteal phase stimulation alone are needed.

Conclusion

Our study emphasizes that follicles entering the anovulatory phase in the follicular phase can be saved by LPS. Although there is no comparable difference in number, oocyte

retrieval in the LPS phase provides clinical benefits for the patient. It also encourages clinicians to undertake additional clinical and laboratory studies that could radically change the approach to ovarian stimulation in the future.

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