Gynecological Endocrinology

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/igye20

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To cite this article: Selmo Geber, Luiza P. Geber, Marcello Valle & Marcos Sampaio (2021): Four consecutive minimal ovarian stimulation (TetraStim) is a feasible alternative to increase the number of oocytes and improve live birth rates in poor responders who do not accept oocyte donation, Gynecological Endocrinology, DOI: 10.1080/09513590.2021.1922887

To link to this article: https://doi.org/10.1080/09513590.2021.1922887

Published online: 23 Jun 2021.

GYNECOLOGICAL ENDOCRINOLOGY
https://doi.org/10.1080/09513590.2021.1922887

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ORIGINAL ARTICLE

Four consecutive minimal ovarian stimulation (TetraStim) is a feasible alternative to increase the number of oocytes and improve live birth rates in poor responders who do not accept oocyte donation

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ABSTRACT

Objective: To present our experience using four consecutive minimal COS (TetraStim) followed by oocyte retrieval and vitrification to increase the number of oocytes in patients with POR for whom oocyte donation is not an option.

Methods: We performed an observational study evaluating 128 poor responders submitted to TetraStim instead of oocyte donation cycles. Patients were submitted to four consecutive minimal COS started at luteal phase, oocyte retrieval, oocyte vitrification/warming, ICSI, endometrial priming and embryo transfer. We evaluated the number of vitrified oocytes, survival rate after warming, fertilization rate, cleavage rate, number of embryos transferred, clinical pregnancy rate, miscarriage rate and live birth rate.

Results: The mean age was 38.1 ± 3.1 years. A total of 791 oocytes were recovered (6.1 ± 2.7/patient), 682 (86.2%) Metaphase II (5.3 ± 2.4/patient) were vitrified, 95.3% survived warming (5.1 ± 2.3/patient), 82% showed normal fertilization after ICSI (4.2 ± 2/patient), 79.2% reached cleavage stage (3.3 ± 1.6/patient), 313 cleavage stage embryos were transferred to 115 patients (2.7 ± 0.7/patient) and 14.7% of the patients had surplus embryos that were vitrified. Clinical pregnancy rate per patient was 31.3% and live birth rate per patient was 22.6%.

Conclusion: To our knowledge this is the first study that demonstrates that TetraStim can be an effective alternative for patients with POR with an indication to perform IVF with donated oocytes, but do not agree to use. TetraStim is a feasible alternative to increase the number of oocytes and embryos and improve pregnancy rates with no dropouts and very low cycle cancelation rate. However, randomized controlled studies must be performed to compare TetraStim with other treatments.

ARTICLE HISTORY
Received 12 January 2021
Revised 13 April 2021
Accepted 24 April 2021
Published online 23 June 2021

KEYWORDS
Poor responders; oocyte donation; TetraStim; minimal stimulation; oocyte vitrification; Bologna criteria; Poseidon

Introduction

One of the most challenging and still unsolved problems related to infertility treatment, that limit its outcomes, is the low number or the absence of oocytes after controlled ovarian stimulation (COS). Poor ovarian response (POR) is associated to low ovarian reserve that might be secondary to advanced maternal age or previous ovarian surgery [1], but can also be observed in some young women with normal ovarian reserve. In these cases, the response might have been affected by the presence of genetic polymorphisms affecting the gonadotropins or their receptors, influencing follicular development, ovarian steroidogenesis and the ovarian response to COS [2]. The incidence of POR ranges from 9–24% and, as these group of individuals is not homogeneous, the pregnancy rate is very low, depending on patient's age and number of oocytes, ranging from 3–14% [3].

In order to standardize the definition of POR, the European Society of Human Reproduction and Embryology (ESHRE) consensus proposed the Bologna criteria in 2011. According to the criteria, a response can be defined as poor when at least two of the following three features present: advanced maternal age; previous poor response, (three or less oocytes after COS); or abnormal ovarian reserve test. However, in the absence of the above criteria, two previous POR following maximal stimulation are enough to classify a patient as a poor responder [4]. Recently, a more detailed stratification of infertility patients with low prognosis in ART was proposed. The Poseidon criteria stratify low prognosis patients into four categories based on a combination of quantitative and qualitative parameters [5]. Regardless of the definition, patients with few oocytes have high cycle cancelation rates, low pregnancy rates and high treatment drop-out rates.

Several strategies have been proposed to improve ovarian response in POR. The most used clinical approaches are the increase of daily gonadotrophin dose, addition of luteinizing hormone or hCG and use of GnRH antagonist regimen [6]. Other strategies consider the addition of adjuvant therapy during COS that include clomiphene citrate, testosterone, dehydroepiandrosterone, letrozole, steroid hormones, growth hormone, coenzyme Q10 [7] and follicular flushing during oocyte retrieval [8]. However, there is still no consensus and the optimal approach to increase the number of oocytes still remains controversial.

Cobo et al. [9] proposed the accumulation of oocytes from several ovarian stimulation cycles using high doses of gonadotrophins to increase the inseminated cohort. The authors concluded that accumulation of oocytes is a successful alternative for low responders as offers comparable success rates to those in

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normoresponders. Recently, Ubaldi et al. [10] proposed a new alternative for POR, doubling the COS within the same cycle. The DuoStim stimulation strategy, combined follicular phase and luteal phase stimulations, using high doses of gonadotrophins, in poor prognosis patients, based on the evidence of the multiple follicular waves within the ovarian cycle. The authors concluded that the use of DuoStim increased the final transferable blastocyst yield and the number of patients with available euploid blastocysts and the final clinical outcomes.

Until now, the only alternative providing actual and consistent results is the use of donated oocytes. However, when this option is not accepted and the couple still seek treatment for conception, the lack of effective treatment alternatives is frustrating. In these cases, we alternatively offered the option to cryopreserve oocytes in order to accumulate and subsequently thaw, inseminate and transfer the embryos. Therefore, the aim of our study is to present our experience using four consecutive minimal controlled ovarian stimulation (TetraStim) followed by oocyte retrieval and vitrification, to increase the number of oocytes in patients with POR for whom oocyte donation is not an option.

Material AND methods

Patients

Patients with previous indication for IVF using oocyte donation due to poor response were included in this prospective observational study. Poor response was defined using the Bologna criteria and Poseidon criteria groups 1a, 2a, 3 and 4. All patients had more than two previous COS with a maximum of three oocytes and/or AMH <0.5 ng/mL. Data was collected from April 2015 to October 2019, in a private IVF center in Brazil. All patients, included in the study, refused to receive donated oocytes and seek for an alternative treatment. All were informed that is was an attempt with no expectation of success due to poor prognosis. After agreement, all signed an informed consent form. An institutional review board approved this study.

Ovarian stimulation and oocyte retrieval

Controlled ovarian stimulation started in the luteal phase (day 19 to day 21) with daily oral intake of 100 mg of Clomiphene Citrate (Clomid, Medley, Brazil) and 5 mg of Letrozole (Letrozol, Eurofarma, Brazil) for 7 days. Cycle phase was confirmed using vaginal serial ultrasound started on the early follicular phase (day 3 to day 5 of the menstrual cycle) and followed until ovulation was confirmed by identification of a corpus luteum. Ovarian response was measured by follicular growth, as monitored by vaginal ultrasound. When a leading follicle reached 13 mm of diameter, patients received GnRH antagonist (Cetrorelix, Cetrotide, Merck, Brazil) for pituitary suppression and 75 IU of recombinant FSH/LH (Pergoveris, Merck, Brazil) per day until the leading follicle reached 17 mm. The use of Clomiphene and Letrozole were suspended when we started FSH/LH. Oocyte maturation was induced with GnRH agonist (0.2 mg triptorelin - Gonapeptyl Daily, Ferring, Brazil) when at least one follicle reached a mean diameter of 17 mm. Oocyte retrieval was performed 36 h later. If an oocyte was not identified in the follicular fluid, 3 ml of HEPES buffered culture medium (Sigma, USA) was injected into the follicle, and the intrafollicular flushing was reaspirated until the oocyte was identified or up to a maximum of five times [8]. Metaphase II oocytes were vitrified 2 h later. One day after oocyte retrieval, all patients started a new ovarian stimulation using Clomiphene and Letrozole until a leading follicle reached 13 mm and patients started GnRH antagonist + FSH/LH until we induced oocyte maturation and a new oocyte retrieval was performed. The same protocol was repeated for the following cycles until oocyte retrieval number four (Figure 1).

Oocyte vitrification and warming

All Metaphase II oocytes included in the study were cryopreserved with Vitrification Kit (Inagmed, Brazil). Briefly, oocytes were placed in an equilibration solution (VI-1) with 7.5% ethylene glycol (v/v), 7.5% dimethyl sulfoxide (v/v) in a HEPES buffered medium, then in a vitrification solution (VI-2) with 15% ethylene glycol (v/v), 15% dimethyl sulfoxide (v/v), 0.5 M of sucrose in a HEPES buffered medium, according to the manufacturer's instructions. Oocytes were placed in an open system straw (Cryo-Inga, Ingamed, Brazil) then submerged into liquid nitrogen. Thawing was performed using a commercial kit (Ingamed, Brazil). Warming kit contained two-step solutions with sucrose 1 M and 0.5 M, in a HEPES buffered medium.

ICSI, embryo culture and transfer

Warmed oocytes were cultured for 2 h before intracytoplasmic sperm injection (ICSI). Approximately 18 h later (day 1) the oocytes were checked for normal fertilization by the presence of two pronuclei. The embryos were kept in culture media (Cleavage, Cook Medical, Australia) at 37 °C in a Petri dish under paraffin oil (Ovoil, Vitrolife, Sweeden) and under a gas phase of 8% CO_2, and were evaluated daily based on standard morphological parameters until transfer [11]. All embryos were transferred in cleavage stage.

Endometrial priming

For endometrial priming, patients started with subcutaneous administration of leuprorelin 3.75 mg (Lectrum, Sandoz, Brazil) on the 2nd or 21st day of the menstrual cycle. When Estradiol

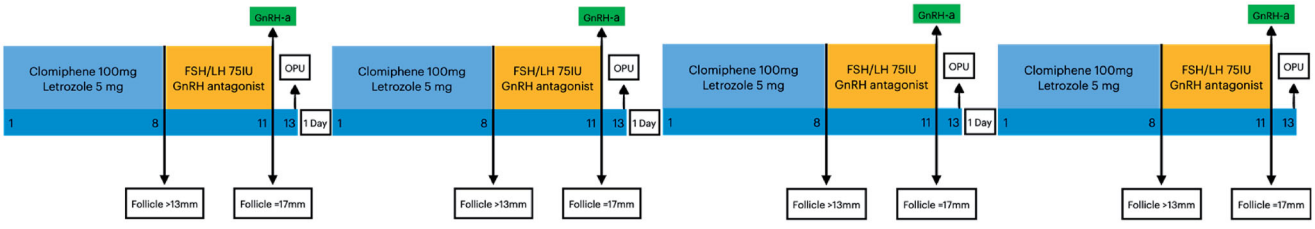

Figure 1. TetraStim protocol for Poor Ovarian Responders. Starts in the luteal phase. GnRH: gonadotrophin-releasing hormone; GnRH-a: GnRH agonist.

concentration was <30 pg/ml and the ultrasound showed an endometrial thickness of <3 mm, pituitary suppression was confirmed and treatment started with estradiol valerate (Primogyna, Bayer, Brazil) 2 mg per day from day 1–5, then the dose was increased to 4 mg per day from day 6–10, and increased to 6 mg per day from day 11. After 15 days, endometrial preparation was confirmed if Estradiol levels were >150 pg/ml and vaginal ultrasound showed an endometrial thickness >7 mm. Vaginal micronized progesterone in gel (Crinone 8%; Merck, Brazil) was started 48 h before when transfer was performed on Day 2 and 72 h before when transfer was performed on Day 3.

Embryo transfer and outcomes

Embryo transfers were performed using a soft transfer catheter (Sydney IVF; Cook – Australia) under abdominal ultrasound guidance. Serum beta-hCG was measured 11 or 12 days after ET. Confirmation of pregnancy was made by vaginal ultrasonography at 2 and 4 weeks after, when a fetal heartbeat was observed. We evaluated the number of vitrified oocytes, survival rate after warming, fertilization rate, cleavage rate, number of embryos transferred, clinical pregnancy rate (presence of gestational sac with fetal heartbeat), miscarriage rate and live birth rate (LBR). The results are presented as mean ± SD and percentage.

Results

All patients had more than two previous COS with a maximum of three oocytes and/or AMH <0.5 ng/mL and were included in the Bologna criteria and Poseidon criteria groups 1a, 2a, 3 and 4. Also, all had a previous indication for IVF using oocyte donation but refused due to personal reasons. The same ovarian stimulation protocol was performed in all patients' cycles. A total of 128 patients were submitted to 512 cycles of consecutive COS cycles and oocyte retrievals (TetraStim) as all patients were submitted to all four cycles. No adverse effects or complications were reported or observed. The mean age was 38.1 ± 3.1 years (range 31–45) and only 6 patients (4.7%) were less than 35 years old.

A total of 791 oocytes were recovered (6.1 ± 2.7 oocytes/patient ranging from 0 to 12) after follicular aspiration and 682 (86.2%) Metaphase II oocytes (5.3 ± 2.4 oocytes/patient ranging from 0 to 12) were vitrified. Only one patient did not have oocytes after TetraStim (0.8%) and 11 patients decided not to perform embryo transfer until this moment, so the oocytes were not warmed (Table 1). Out of 621 vitrified mature oocytes, 592 (95.3%) survived warming (5.1 ± 2.3 oocytes/patient ranging from 1 to 12). All oocytes were inseminated by ICSI and 486 (82%) showed normal fertilization (4.2 ± 2 oocytes/patient ranging from 1 to 10). From the 486 2 pronuclei stage, 385 (79.2%) reached cleavage stage (3.3 ± 1.6 embryos/patient ranging from 1 to 6) (Table 1).

One patient decided to submit the embryos to biopsy followed by PGT-A and did not have euploid embryos to transfer. Therefore, the embryo transfer cancelation rate was 0.9% (1 out of 116 who warmed oocytes). A total of 313 cleavage stage embryos were transferred to the 115 patients (2.7 ± 0.7 embryos/patient) and the number of transferred embryos ranged from 1 to 3. A total of 17 patients had surplus embryos that were vitrified (14.7%). Overall clinical pregnancy rate per patient was 31.3% ($n = 36$) with a live birth rate per patient of 22.6% ($n = 26$). There were no multiple pregnancies (Table 2).

Table 1. Clinical results and Laboratory outcomes after TetraStim, oocyte vitrification, warming and ICSI in Poor Ovarian Responders.

Patients (*n*)	128
Stimulation cycles (*n*)	512
Oocyte retrievals (*n*)	512
Age	
Mean	38.1 ± 3.1
Range	31–45
Oocytes (*n*)	791
Mean/patient	6.1 ± 2.7
Range	0–12
Metaphase II (*n*)	682
Mean/patient	5.32 ± 2.4
Range	0–12
Patients with ≥ 1oocyte	127 (99.2%)
Oocyte survival rate	95.3%
n/warmed	592/621
Mean/patient	5.1 ± 2.3
Range	1–12
Fertilization rate	82%
n/inseminated	486/592
Mean/patient	4.2 ± 2
Range	1–10
Cleavage rate	79.2%
n/fertilized	385/486
Mean/patient	3.3 ± 1.6
Range	1–6

Table 2. Clinical outcomes after TetraStim, oocyte vitrification, warming, ICSI and Embryo Transfer in Poor Ovarian Responders.

Patients (*n*)	115
Transferred embryos (*n*)	313
Mean/patient	2.7 ± 0.7
Patients with frozen embryos	17 (14.7%)
Clinical pregnancy rate	31.3%
Miscarriage rate	27.7%
Live birth rate	22.6%

Discussion

The number of oocytes is a crucial factor to obtain satisfactory outcomes during IVF treatment. Therefore, in the group of patients with POR there is a compromise in the pregnancy rate due to the reduced number or even absence of oocytes. Moreover, there is no ideal alternative to increase the number of oocytes and pregnancy rate except the use of donated oocytes. To our knowledge this is the first study that demonstrates that the use of four consecutive minimal controlled ovarian stimulation (TetraStim) is a feasible alternative to increase the number of oocytes and improve pregnancy rates of POR that do not accept the use of donated oocytes.

We analyzed a group of patients with POR matching the Bologna criteria and Poseidon criteria groups 1a, 2a, 3 and 4 and all had a previous indication to receive donated oocytes. This strict limitation in the inclusion criteria, makes this a very homogeneous group. As expected, the mean age was >38 years, since age-related poor ovarian reserve is the most common cause of POR. As only 6 patients were <35 years and all had the same POR criteria we did not divide in age groups for comparison. As we did not perform a comparative study, we did not calculate the sample size.

As previous studies reported higher number of retrieved Metaphase II oocytes after luteal phase COS for normal and low responders, we started TetraStim at luteal phase [12–15]. Also, as the three following COS were performed immediately after oocyte retrieval, they also started at luteal phase. Thus, we understand that starting at luteal phase not only did not

compromise, but also might have contributed to our results. Moreover, the immediate start of a new COS after oocyte retrieval makes the treatment faster than as it took approximately two months for patients to finish TetraStim instead of the expected four months if we had used COS with two weeks interval after retrieval. Also, the previous knowledge of a continuous stimulation, might have a positive psychological impact, which can be verified by the absence of patients' drop out.

The alternative approach of oocyte accumulation for POR was firstly described by Cobo et al. [9]. The authors used high doses of gonadotrophins in nonconsecutive regular follicular phase COS and described comparable success rates when compared to normoresponders. More recently, Kuang et al. [16] used Clomiphene Citrate and Letrozole, but associated to high doses of gonadotrophins for two consecutive COS in poor responders. Ubaldi et al. [10] and Alsbjerg et al. [14] also described the use of two consecutive COS (DuoStim) for patients with low ovarian reserve, using high doses of gonadotrophins. In all the three above studies, the authors did not cryopreserve the oocytes, but the embryos with high pregnancy rates for this population. In our study we used Clomiphene Citrate and Letrozole followed by low dose gonadotrophin for few days after starting GnRH antagonist and we vitrified all Metaphase II oocytes for subsequent ICSI after warming. As previously demonstrated, the use of minimal/mild ovarian stimulation is as effective as conventional ovarian stimulation in POR [17, 18] and should be considered a more cost-effective approach for poor responders in an oocyte–embryo accumulation strategy [19]. Therefore, we reduced treatment cost by using a minimal/mild COS, without compromising ovarian response, as all were poor responders, and by performing only one ICSI after warming of all oocytes. Also, as we froze oocytes and not embryos, we only performed ICSI once, reducing the costs of treatment. Moreover, the use of oral stimulants instead of injectables for 7 days, makes the treatment easier for patients submitted to TetraStim. We associated Clomiphene Citrate and Letrozole as they have different mechanisms of action which could potentiate the effects on ovarian stimulation.

As it has been previously demonstrated for fresh oocytes [20] that patients with suboptimal response (4–9 oocytes) had similar live birth rates when compared to those with high and normal response (>9 oocytes) and higher than low responders (1–3 oocytes), we decided to perform four consecutive COS to reach a sufficient number of oocytes (>4) and embryos to achieve transfer with higher pregnancy rates than the observed in POR. As the mean number of retrieved oocytes was 6.1 (5.3 Metaphase II) and only one patient (0.8%) did not have oocytes after TetraStim, that is less than the previously described for POR patients [10,14] we considered to have succeeded in this decision.

Vitrification of oocytes is one of the most important advances in the recent history of fertility preservation mainly due to the high survival rates. The oocyte survival rate observed in our study (95%) was similar to the described in previous published for non-POR young patients [21–23]. So, it can be assumed that POR patients do not have a worse prognosis than the observed in normal responders for vitrification. Fertilization rate (82%) was similar to the observed with fresh oocytes after ICSI with non-male factor infertility diagnosis [24] and higher to the previously published with vitrified/warmed oocytes in POR and non-POR patients [9,22,23]. Cleavage rate observed in our study (79%) was in accordance to the described by Cobo et al. [9] in a group of POR patients submitted to accumulation of oocytes and by Rienzi et al. [23] for patients that received donated vitrified oocytes. This result allowed the availability of 3.3 embryos for transfer (range = 1–6), for each patient.

Among all patients who have had oocytes (99.2%), all who decided to have a transfer had available embryos. Only one patient did not have an embryo transfer as she requested PGT-A and none of them was euploid. Therefore, the cancelation rate (1%) was lower than the previously described for POR patients at the same age that accumulated oocytes [9,14,16] and the described for normal responders after elective fertility preservation [21]. The mean number of transferred embryos (2.7/patient) was considered optimal as Kamath et al. [25] described no differences in the LBR when compared patients who had 2 embryos with those receiving 3 or 4 embryos. Moreover, 14.7% of these patients had surplus embryos that were vitrified for further embryo transfer, increasing the cumulative pregnancy rate, which is high and of clinical importance for this group of patients. The use of artificial endometrial priming in all POR patients was not a matter of concern as we have previously demonstrated that Freeze-all strategy using artificial priming, compared with fresh ET, has no impact on IVF outcome among poor responders [3].

The observed clinical pregnancy rate per patient (31.3%) and the live birth rate per patient (22.6%) were higher than the described for low responders (6–12%) [3,8,26–29]. However, when we compared our results to the described for oocyte/embryos accumulation for low responders, we observed similar results (20–30%) [9,10,14,16]. The observed miscarriage rate (27.7%) can be explained by the advanced maternal age of our group of patients.

A limitation of this study was that we did not to compare with other different treatment strategy. However, another COS approach was not offered as all patients had Bologna and Poseidon groups 1a, 2a, 3 and 4 criteria and previous indication for oocyte donation. As a future alternative we could consider the use of oral progesterone to replace GnRH for pituitary suppression in order to reduce the cost and the use of injectables, making TetraStim less stressful.

In conclusion, our study demonstrated for the first time that the use of TetraStim can be an effective alternative for patients with POR with an indication to perform IVF with donated oocytes, but do not agree to use. The results show that four consecutive minimal/mild ovarian stimulation starting at luteal phase is a feasible alternative to increase the number of oocytes and embryos and improve pregnancy rates with no dropouts and very low cycle cancelation rate. However, randomized controlled studies must be performed to compare TetraStim with other treatments.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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