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Research Article

Mild Ovarian Stimulation Using the Aromatase Inhibitor Letrozole (LTZ) is a Good Stimulation Protocol for to Obtain Euploid Blastocysts for Advanced Maternal Age Women

Koji Nakagawa[1]*, Takashi Horikawa[1], Keisuke Shiobara[1], Shunsuke Ishiyama[1], Hisayo Kataoka[1], Katsuki Nakao[1], Yuko Ojiro[1], Keiji Kuroda[2], Satoru Takamizawa[1] and Rikikazu Sugiyama[1]

[1]Center for Reproductive Medicine and Implantation Research, Sugiyama Clinic Shinjuku, Tokyo, Japan

[2]Center for Reproductive Medicine and Endoscopy, Sugiyama Clinic Marunouchi, Tokyo, Japan

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***Corresponding author**: Koji Nakagawa, MD, PhD, Center for Reproductive Medicine and Implantation Research, Sugiyama Clinic Shinjuku, 1-19-6, Nishi-Shinjuku, Shinjuku-ku, 160-0023, Tokyo, Japan, E-mail; koji@sugiyama.or.jp

ORCiD: https://orcid.org/0000-0003-0874-5894

Keywords: Letrozole (TTZ); Euploid; Advanced maternal age; Clomiphene citrate (CC); Progestin

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Abstract

Purpose: Mild Ovarian Stimulation (MOS) is an effective form of Assisted Reproductive Technology (ART) for Advanced Maternal Age (AMA) women. Taking either Clomiphene (CC) or Letrozole (LTZ) with MOS reduces the amount of gonadotropin that must be used. It has remained uncertain, however, whether LTZ or CC is more effective in combination with MOS. Therefore, we evaluated the choice of combination of either CC or LTZ with MOS and quantified the obtainment of euploid blastocysts for AMA women.

Methods: This retrospective study was conducted between January 2020 and April 2021. In this study, we stimulated 286 women with MOS, 153 of these women used CC (CC group) and 133 were given LTZ (LTZ group). All women took either 100 mg of CC or 5 mg of LTZ daily for 7 days between MC 3 and MC 9, and 225 IU of recombinant-FSH were administered on MC 3, MC 5, MC 7, and MC 9. The euploid rates were compared between these two groups. Among them, the women who were ≥ 40 years old were divided into two groups, the CC-O40 group (n = 61) and LTZ-O40 group (n = 54), and the euploid rates were evaluated.

Results: For AMA women, the blastocyst formation rate in the LTZ-O40 group (58.5%) was significantly higher than that in the CC-O40 group (46.6%, $p < 0.05$). The euploid rate in the LTZ group was 53.4%, which was significantly higher than that in the CC group (38.0%, $p < 0.05$). The AMA euploid rate in the LTZ-O40 group was 40.5%, which also was significantly higher than that in the CC-O40 group (16.7%, $p < 0.05$).

Conclusions: For AMA women, a regimen of LTZ combined with MOS is the most effective ovarian stimulation method for obtaining euploid blastocysts.

Introduction

In Assisted Reproductive Technology (ART), obtaining many morphologically good blastocysts is a shortcut to success, because the clinical pregnancy rate of blastocyst transfer with a morphologically good blastocyst is expected to be ≥ 40% among women who are less than 40 years of age [1], and this is significantly higher than that of cleavage-stage embryo transfer [2]. This is why either long or short protocols of Gonadotropin-Releasing Hormone (GnRH) agonists with high doses of gonadotropins have been popular for the past two decades [3]. However, these protocols force patients to bear physical and economic burdens [4], which is why we developed a new ovarian stimulation protocol using

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mild stimulation with Clomiphene Citrate (CC) [5]. This mild and minimal ovarian protocol has become the first choice among patients who wish to receive ART treatment, and this is particularly true for Advanced Maternal Age (AMA) patients [6]. Currently, we prefer to use an aromatase inhibiter instead of clomiphene citrate in this mild stimulation protocol to prevent the occurrence of Ovarian Hyperstimulation Protocol (OHSS) and to reduce the physical symptoms following Oocyte Pick-Up (OPU). The use of aromatase inhibiter Letrozole (LTZ) is timed to inhibit the endogenous estradiol production from ovaries with multiple follicular growth in conjunction with the occurrence of the maturation trigger. This could result in cerebral pontine infraction (pons) for patients, but LTZ could not suppress the LH surge or LH rise in estrogen-derived positive feedback. When we select this protocol (MOS with aromatase inhibiter), we must pay attention to either a surge or an early rise in Luteinizing Hormone (LH), and in some cases, a Gonadotropin-Releasing Hormone (GnRH) antagonist must be used to prevent LH surge. When using a GnRH antagonist, however, the amount of gonadotropin used for ovarian stimulation must be increased.

The blastocyst formation rate is known to decline in AMA women, and the rates of morphologically good blastocyst delivery are particularly low for AMA women who are more than 40 years of age [7,8]. Preimplantation Genetic Testing for Aneuploidy (PGT-A) has been clinically applied since 2019 in Japan [9]. This technique has shown good results even in AMA women when using euploid blastocysts for transfer [10]. For women, obtaining blastocysts for biopsy is sometimes difficult, however, and an ovarian stimulation protocol for efficiently obtaining morphologically good blastocysts could be practical.

Progestin-Primed Ovarian Stimulation (PPOS) was first introduced as one of the stimulation protocols for ART treatment by Kaung Y and co-workers in 2015 [11]. Their purpose for using progestin during ovation stimulation was to prevent a premature LH surge during ovarian stimulation by reducing cycle cancellation and thereby promoting the safety of patients [12]. They applied their data to clinical field results showing that progesterone secreted from the corpus luteum suppressed pulsatile Gonadotropin-Releasing Hormone (GnRH) in the hypothalamus. As a consequence, LH secretion from the pituitary gland was suppressed, and estradiol (E2)-induced positive feedback effects were prevented [11]. These researchers reported that, with these precautions in place, the incidence of premature LH surge was seen in only 0.7% of the patients, but the amount of HMG doses was significantly higher than that in the control group stimulated by GnRH-agonist short protocols.

Therefore, we intended to modify this PPOS protocol to reduce the HMG dosage. Our new protocol combined mild stimulation with the administration of progestin to prevent premature LH surge, and we prefer this new protocol as ovarian stimulation with a Later Administration of Progestin (MS-LAP). The purpose of this study was to evaluate this MS-LAP protocol with LTZ and compare it with MS-LAP using CC with regard to the rates of morphologically good blastocysts (euploid) that are produced.

Materials and methods

Study design

This single-center, cross-sectional study was conducted at Sugiyama Clinic Shinjuku, Tokyo, Japan between January 2020 and April 2021. For this study, we recruited patients who had opted to receive IVF treatment via MS-LAP as an ovarian stimulation protocol during the study period. A total of 286 patients who had undergone ART treatment participated in the present study. Written informed consent was obtained from all patients, and this study was approved by the Institutional Review Board of the Sugiyama Clinic Shinjuku (2019-01). The indications for ART treatment included tubal factor infertility (17.5%), unexplained infertility (40.4%), endometriosis (8.9%), and male-factor infertility (33.2%). All male-factor infertility cases were treated by Intracytoplasmic Sperm Injection (ICSI). A flowchart of the patient selection process is shown in Figure 1. Of the 286 patients who received ovarian stimulation with MS-LAP for ART treatment, 153 were given Clomiphene Citrate (CC) as an oral medicine for ovarian stimulation (CC group), and the others (n = 133) received the aromatase inhibitor Letrozole (LTZ group). The ART outcomes were compared among these two groups. Among them, patients who were ≥ 40 years of age were placed into either the CC-O40 group (n = 61) or the LTZ-O40 group (n = 54), and the ART outcomes were compared among these two groups.

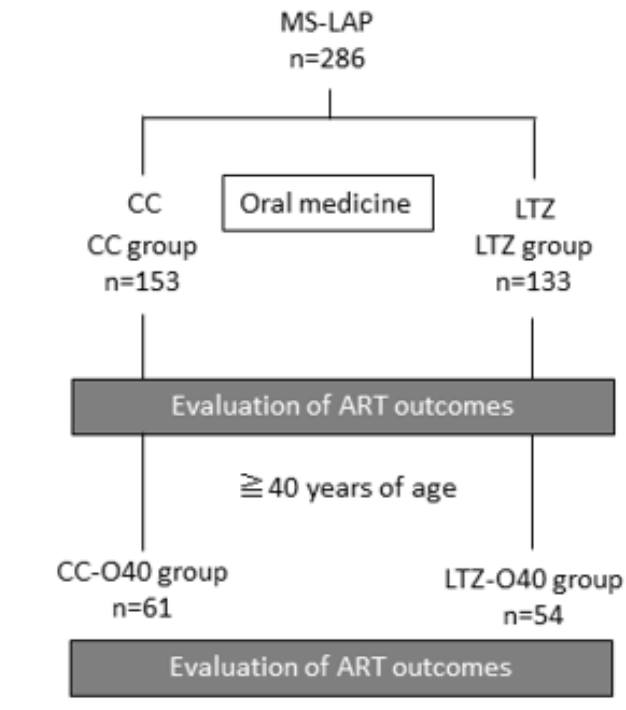

Figure 1: This is s flow chart of the patient selection process. Of the total 288 patients who received ovarian stimulation with MS-LAP for ART treatment, 153 were given CC as an oral medicine for ovarian stimulation (CC group), and the others (n = 133) received the aromatase inhibitor letrozole (LTZ group). The ART outcomes were compared among these two groups. Among them, patients who were ≥ 40 years of age were placed into either the CC-O40 group (n = 61) or the LTZ-O40 group (n = 54), and the ART outcomes were compared among these two groups.
MS-LAP: Mild Stimulation Protocol with a Later Administration of Progestin; CC: Clomiphene Citrate; LTZ: Letrozole.

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Ovarian stimulation protocol of MS-LAP

All treatment cycles involved our mild stimulation protocol as follows. On the 3rd day of their menstrual cycle or withdrawal bleeding (MC 3), all patients received transvaginal ultrasound to check for the presence of residual follicles, endometrial cysts, and ovarian cysts; at this point, they also were measured for estradiol (E2), follicle-stimulating hormone (FSH), and Luteinizing Hormone (LH) levels. When E2 levels were elevated to more than 100 pg/ml or when the presence of residual follicles (≥ 11 - 12 mm in diameter) was detected, although the expected number of developed follicles could have been low due to unsynchronized follicle growth, then this cycle was canceled. When an elevation in the FSH level (≥ 15 IU/L) was confirmed, the gonadotropin dose was increased. Anti-Mullerian Hormone (AMH) values for all cases also were measured before starting ovarian stimulation.

Hormonal assays were performed as follows. The E2, FSH, and LH levels were measured on MC 3 using commercially available CLEIA kits (AIA-PackCL®; TOSOH CORORATION, Tokyo, Japan). The E2, LH, and progesterone levels also were measured on MC10 using the same ERISA kits. The AMH values were measured using a commercially available ELISA kit (VIDAS AMH Assay kit, Biomérieux Japan, Tokyo, Japan).

The ovarian stimulation protocol for MS-LAP appears in Figure 2. All patients took either 100 mg of clomiphene citrate (Clomid®, Fuji Pharma, Tokyo; CC group) or 5 mg of the aromatase inhibitor letrozole (Letrozole®, Fuji Pharma, Tokyo; LTZ group) daily for 7 days between MC3 and MC9 of their menstrual cycle, and 225-300 International Units (IU) of recombinant-FSH (Gonal-F®, Merk Serono, Tokyo) were administered on MC 3, MC 5, MC 7, and MC 9 depending on the FSH levels on MC 3. Progestin administration (10 mg of medroxyprogesterone acetate; Hysron®, Kyowa-Kirin, Tokyo) was begun on MC 8 twice a day until the day of maturation trigger. On MC 10, the number of grown follicles that reached ≥ 20 mm in diameter were counted, and hormonal levels of E2, LH, and progesterone were also measured. The appearance of 3 or more well-developed follicles (≥ 20 mm in diameter) tripped a dual maturation trigger for the administrations of 250 µg of recombinant human chorionic gonadotropins (rec-hCG; Ovidrel®, Merk Serono, Tokyo) and 300 ug of GnRH agonist nasal spray (Buserequr®, Fuji Pharma, Tokyo) twice with 30-minute intervals; oocyte-pick up (OPU) was performed 35-36 hours later. If adequate follicle growth was not confirmed by MC 10, additional rec-FSH (225 or 300 IU per day) or either clomid® or letrozole® was administered as needed, which was repeated 2 days later (MC 12). Then, both the transvaginal ultrasound examination and hormonal measurement were repeated.

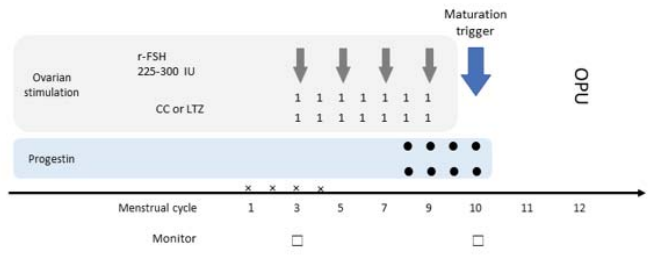

Figure 2: All patients took either 100 mg of clomiphene citrate (Clomid®, Fuji Pharma, Tokyo; CC group) or 5 mg of the aromatase inhibitor letrozole (Letrolzole®, Fuji Pharma, Tokyo; LTZ group) daily for 7 days between MC3 and MC9 of their menstrual cycle, and 225-300 International Units (IU) of recombinant-FSH (Gonal-F®, Merk Serono, Tokyo) were administered on MC3, MC5, MC7, and MC9 depending on the FSH levels on MC 3. Progestin administration (10 mg of medroxyprogesterone acetate; Hysron®, Kyowo-Kirin, Tokyo) was begun on MC 8 twice a day until the day of maturation trigger. On MC 10, the number of grown follicles that reached ≥ 20 mm in diameter were counted, and hormonal levels of E2, LH, and progesterone also were measured. The appearance of 3 or more well-developed follicles (≥ 20 mm in diameter) tripped a dual maturation trigger for the administrations of 250 µg of recombinant human chorionic gonadotropins (rec-hCG; Ovidrel®, Merk Serono, Tokyo) and 300 ug of GnRH agonist nasal spray (Buserequr®, Fuji Pharma, Tokyo) twice with 30-minute intervals; OPU was performed 35-36 hours later. CC: clomiphene citrate, LTZ: letrozole, OPU: oocyte pick-up.

IVF procedure and blastocyst culture

OPU was performed under transvaginal ultrasound with or without general or local anesthesia. The retrieved oocytes were inseminated using either conventional insemination or Intracytoplasmic Sperm Injection (ICSI) due to the results of either the sperm preparation or a patient's characteristics. Piezo-ICSI was adapted for all ICSI cases [13]. After fertilization was confirmed, the fertilized oocytes were continuously cultured to the blastocyst stage under mixed-gas conditions (4% O_2, 6% CO_2, and 89% N_2) [14] in a time-lapse incubator (Geri™, Genea Biomedx, VIC, Australia). The culture medium GEMS GERI MEDIUM™ (Genea Biomedx, VIC, Australia) was used, and all embryos were cultured individually immediately after insemination and at the times of blastocyst morphological evaluations. Some patients wished to receive preimplantation genetic testing for aneuploidy (PGT-A) due to repeated implantation failures or a recurrence of pregnancy loss. When the blastocysts reached ≥ 4BB according to Gardner's classification [15], a TE biopsy was performed. All blastocysts, either with or without TE biopsy, were cryopreserved between 120 and 150 hours following insemination.

TE biopsy, NGS, and blastocyst cryopreservation

The procedures for Trophectoderm (TE) biopsy and NGS in our laboratory were recently published [16], and briefly described. The TE biopsy performed in our laboratory involved a mechanical dissection technique without laser-assisted hatching. Under continuous observation using time-lapse monitoring, the expanded blastocysts that possessed an adequate number of TE cells were biopsied for use in PGT-A. First, an infrared diode laser (Saturn 5™ Active, Cooper Surgical, CT, USA) was used to create a small hole in the zona pellucida, which induced blastocele collapse. Next, a biopsy pipette (Kitazato, Shizuoka, Japan) was inserted through that small hole into the perivitelline space, and TE cells were aspirated. After TE aspiration, around 5-10 TE cells were obtained by using a mechanical blunt-dissection technique [17]. These procedures were performed by applying micromanipulation equipment to droplets of PGD biopsy medium (Global, LifeGlobal, USA). All biopsied TE cells were washed twice in sterile Phosphate-Buffered Saline (PBS) solution containing 1% Polyvinylpyrrolidone (PVP) solution, and these washed

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cells were expelled into a 0.2 ml PCR tube with 2.5 µL of Phosphate-Buffered Saline (PBS) at a temperature -20 °C until they could be amplified. All biopsy samples were sent to the KITAZATO BIOLABORATORY in Tokyo) where Whole-Genome Amplification (WGA) could be performed for analysis via Next-Generation Sequencing (NGS). WGA was accomplished using the Sureplex DNA Amplification System (Illumina, CA, USA), and NGS was performed using the Miseq System (Illumina, CA, USA). The chart analysis was performed using BlueFuse Multi Software (Illumina, CA, USA) [18]. Following the biopsy, all blastocysts were cryopreserved using the vitrification method [19,20].

Clinical outcomes

The main outcome of the present study was to increase the proportion of morphologically good blastocysts and to compare the outcomes when using two similar two types of ovarian stimulation protocols: CC or LTZ. Blastocysts were defined as full blastocysts and blastocoel completely filling the embryo (Gardner's expansion grade ≥ 3), and morphologically good blastocysts were defined by Gardner's classification: ≥ 4BB. The second endpoint was the euploid rate. The biopsied blastocysts were judged as euploid, mosaic, or aneuploid. The blastocysts showing 20% or less of mosaicism or segmental mosaicism were judged to be euploid, and the remainders were either aneuploidy or mosaic [21]. These parameters were applied to both the CC-O40 and LTZ-O40 groups.

Statistical analysis

The data were statistically analyzed using either Fisher's exact test or a non-parametric test. A p - value of < 0.05 was considered statistically significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, EZR is a modified version of R commander, which was designed to add statistical functions that are frequently used in biostatistics [22].

Results

Among all patients, 153 were stimulated by mild stimulation protocol with CC (CC group), and the others (n = 133) were subjected to mild stimulation with LTZ (LTZ group). The backgrounds of the CC and LTZ protocols appear in Table 1. The average age of the patients in the CC and LTZ groups were 38.3 ± 4.1 and 37.8 ± 4.5 (years, mean ± S.D.), and no significant differences were observed (p = .84). The proportion of the patients who were ≥ 40 in the CC group was 39.9%, which was similar to that in the LTZ group (40.6%, p = .90). The AMH values between these two groups were comparable (3.3 ± 2.7 ng/ml in the CC group and 3.3 ± 3.6 ng/ml in the LTZ group, respectively, p = .96). The antral follicle count (AFC) in the CC group (9.2 ± 4.8), however, was significantly higher than that in the LTZ group (7.0 ± 3.7, p < 0.001). The basal FSH level in the CC group (10.0 ± 3.9 IU/L) was also significantly higher than that in the LTZ group (9.2 ± 2.9, p = .046). The basal LH and E2 levels in the CC group, however, were comparable to those in the LTZ group.

The ART outcomes for all ages in both the CC and LTZ groups are quantified in Table 2. The number of retrieved oocytes in the CC group was 7.5 ± 5.3 (mean ± S.D.), which was significantly higher than that in the LTZ group (6.3 ± 4.7, p = .042). The proportions of ICSI cases in the CC and LTZ groups were 51.7 and 66.2%, respectively, which represent a significant difference (p = .0068). The fertilized and developed oocytes in the CC group averaged 5.0 ± 4.2 and 4.9 ± 4.2, respectively, and these were significantly higher than those in the LTZ groups (4.0 ± 3.3, p = .013, and 3.8 ± 3.2, p = .019, respectively). The numbers of blastocysts (2.8 ± 3.0) and morphologically good blastocysts (MGB; 1.8 ± 2.4) in the CC groups were similar to those in the LTZ groups (2.4 ± 2.7, p = .18, and 1.4 ± 2.0, p = .018, respectively). The formation rates for blastocysts and good blastocysts in the CC group were 55.8 and 35.5%, respectively, which was comparable to that for the LTZ group (61.2%, p = .063, and 36.7%, p = .68, respectively).

Table 3 lists the ART outcomes between the CC-O40 and LTZ-O40 groups. The averages for age, number of retrieved oocytes, fertilized oocytes, and developed oocytes between

Table 1: Background of the patients using MS-LAP with CC or LTZ among all age groups.

	CC group	LTZ group	p value
Patients, n	153	133	
Age, years#	38.3 ± 4.1	37.8 ± 4.5	0.84
Age ≥ 40 years, n (%)	61 (39.9)	54 (40.6)	0.90
AMH, ng/mL #	3.3 ± 2.7	3.3 ± 3.6	0.96
AFC, n#	9.2 ± 4.8	7.0 ± 3.7	< 0.001
Basal FSH, IU/L#	10.0 ± 3.9	9.2 ± 2.9	0.046
Basal LH, IU/L#	5.0 ± 2.4	4.7 ± 2.0	0.26
Basal E2, pg/mL#	35.0 ± 18.3	40.8 ± 18.0	0.06

MS-LAP: Mild Ovarian Stimulation with a Later Administration of Progestin; CC: Clomiphene Citrate; LTZ: Letrozole; AMH: Anti-Mullerian Hormone; AFC: Antral Follicle Count

Table 2: The ART outcomes of all age categories between CC and LTZ groups.

	CC group	LTZ group	p value
Patients, n	153	133	-
Retrieved oocytes, n# (total)	7.5 ± 5.3 (1,148)	6.3 ± 4.7 (835)	0.042
Degenerated oocytes, n# (total)	0.27 ± 0.63 (41)	0.20 ± 0.50 (27)	0.34
Insemination, n [ICSI/IVF]	591/491	510/298	0.00023
ICSI cases, n (%)	77 (51.7)	88 (66.2)	0.0068
Fertilized oocytes, n# (total)	5.0 ± 4.2 (747)	4.0 ± 3.3 (515)	0.013
Developed oocytes, n# (total)	4.9 ± 4.2 (722)	3.8 ± 3.2 (504)	0.019
Blastocysts, n# (total)	2.8 ± 3.0 (417)	2.4 ± 2.7 (315)	0.18
Blastocyst formation rate, %	55.8	61.2	0.063
Morphologically good blastocysts, n# (total)	1.8 ± 2.4 (265)	1.4 ± 2.0 (189)	0.18
Good blastocyst rate, %	35.5	36.7	0.68

#mean ± Standard deviation.
Blastocyst formation rates were calculated according to the number of blastocysts produced by the number of fertilized oocytes.
A good blastocyst rate was defined by the number of morphologically good blastocysts produced by the number of fertilized oocytes.

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these two groups were similar with no significant differences. The number of blastocysts in the CC-O40 group was 1.7 ± 1.8, which was similar to that in the LTZ-O40 group (1.7 ± 1.8, p = .88). However, the blastocyst formation rate in the LTZ-O40 group (58.5%) was significantly higher than that in the CC-O40 group (46.6%, p = .028), but the good blastocyst rate between these two groups was similar (23.3% in the CC-O40 group and 30.8% in the LTZ-O40 group, respectively, p = .12).

In the CC and LTZ groups, 39 and 40 patients received TE biopsy for PGT-A, whereas 18 and 19 in the CC-O40 and LTZ-O40 groups also received TE biopsy. The results of PGT-A in the CC and LTZ groups are summarized in Table 4. The TE biopsy rate in the CC group was 13.4%, which was lower than that in the LTZ group (17.1%) and there was no significant difference (p = .0768). The euploid rate in the LTZ group was 54.5%, and this was significantly higher than that in the CC group (38.0%, p = .023). When AMA was considered, the biopsy rate in the LTZ-O40 group was 23.3%, which was significantly higher than that in the CC-O40 group (13.7%, p = .02), and the euploid rate in the LTZ-O40 group was 40.5%, which also was higher than that in the CC-O40 group (16.7%, p = .037, Table 5).

Discussion

A recent report from Japan [23] indicates that the clinical pregnancy rate per single thawed embryo transfer (180,176 cycles) is 37.0%, which is significantly higher than the national rate for single fresh embryo transfer (26,463 cycles, 21.1%), and this is why the freeze-all policy is has become popular in our country. Mild ovarian stimulation combined with oral ovulation-inducing drugs such as clomiphene citrate or letrozole has become a popular protocol for the prevention of Ovarian Hyperstimulation Syndrome (OHSS). This protocol uses effectively endogenous GnRH pulses, which makes it possible to reduce the amount of gonadotropin used without requiring daily administration. However, since no drugs are used to suppress ovulation, there is always the risk of an early rise in LH with the resultant ovulation before oocyte retrieval. GnRH antagonists are frequently used as a method to prevent an early rise in LH from the ovarian stimulation of ART treatment, and although antagonists are a fast-acting means of ovulation suppression, they are relatively expensive and require self-injection, which increases the burden on patients. There is a need for a simple, inexpensive, and more effective method to suppress both the early rise in LH and ovulation.

The PPOS protocol was originally reported by Kong and co-workers [11] in 2015, and this protocol has become the mainstream method for ovarian stimulation protocol in ART treatment in Japan. This protocol is a unique method wherein administered progestin suppresses GnRH pulses from the hypothalamus [24], which allows avoidance of an early rise in LH and ovulation before oocyte retrieval. In the original PPOS protocol, administration of the progestin preparation precedes the start of gonadotropins, which necessitates a longer duration of progestin administration. As a result, endogenous GnRH pulses are reduced and fewer are then available for ovarian stimulation so the use of gonadotropin must be increased. In the PPOS protocol, progestin was used to prevent an early rise

Table 3: The ART outcomes between CC-O40 and LTZ-O40 groups.

	CC-O40 group	LTZ-O40 group	p value
Patients, n	61	54	
Age, years#	42.2 ± 2.1	42.2 ± 1.7	0.97
Retrieved oocytes, n# (total)	5.7 ± 3.7 (345)	5.2 ± 2.9 (282)	0.49
Degenerated oocytes, n# (total)	0.18 ± 0.43 (11)	0.26 ± 0.56 (14)	0.39
Fertilized oocytes, n# (total)	3.6 ± 2.5 (219)	2.9 ± 1.8 (159)	0.12
Developed oocytes, n# (total)	3.5 ± 2.4 (212)	2.9 ± 1.8 (155)	0.14
Blastocysts, n# (total)	1.7 ± 1.8 (102)	1.7 ± 1.7 (93)	0.88
Blastocyst formation rate, %	46.6	58.5	0.028
Morphologically good blastocysts, n# (total)	0.84 ± 1.16 (51)	0.91 ± 1.15 (49)	0.74
Good blastocyst rate, %	23.3	30.8	0.12

#mean ± Standard deviation
Blastocyst formation rates were calculated by the number of blastocysts produced by the number of fertilized oocytes.
A good blastocyst rate was defined by the number of morphologically good blastocysts produced by the number of fertilized oocytes.

Table 4: The outcomes of PGT-A between the CC and LTZ groups.

	CC group	LTZ group	p
Patients performed PGT-A, n	39	40	-
Age, years#	38.5 ± 4.8	38.3 ± 4.2	0.873
TE biopsy, n	100	88	-
TE biopsy rate*, % (n/n)	13.4 (100/747)	17.1 (88/515)	0.0768
Euploid, n (median[range])	38 (1 [0-4])	48 (1 [0-5])	0.423
Euploid rate**, %	38.0	54.5	0.0231
Mosaic, n (median[range])	15 (0 [0-2])	9 (0 [0-2])	0.180
Aneuploid, n (median[range])	47 (1 [0-4])	31 ([0-3])	0.036

#mean±S.D., ## median [range]
*TE biopsy rates were calculated by dividing the number of blastocysts that received TE biopsy by the number of fertilized oocytes.
**The euploid rates were calculated by dividing the number of euploid blastocysts by the number of biopsied blastocysts.

Table 5: The outcomes of the PGT-A between flexible PPOS with CC or Ai among the advanced maternal age patients (≥ 40 years of age).

	CC-O40 group	LTZ-O40 group	p value
Patients receiving PGT-A, n	18	19	
Age, years	42.2 ± 1.8	42.1 ± 1.7	0.767
TE biopsy, n	30	37	
TE biopsy rate, %	13.7 (30/219)	23.3 (37/159)	0.0201
Euploid, n (median[range])	5 (0 [0-1])	15 1 [0-2]	0.024
Euploid rate, %	16.7	40.5	0.037
Mosaic, n (median[range])	3 (0 [0-1])	5 (0 [0-2])	0.688
Aneuploid, n (median[range])	22 (1 [0-3])	17 (1 [0-3])	0.263
Aneuploid rate, %	73.3	48.6	0.0487

#mean ± S.D., ## median [range]
*TE biopsy rates were calculated by dividing the number of blastocysts that received TE biopsy by the number of fertilized oocytes.
**The euploid rates were calculated by dividing the number of euploid blastocysts by the number of biopsied blastocysts.

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in LH and ovulation before the occurrence of the maturation trigger. Therefore, it is unnecessary to use progestin when beginning ovarian stimulation. According to this logic, the use of progestin during the first half of ovarian stimulation would be unnecessary, so progestin administration was begun on the 6th day following the start of our mild ovarian stimulation in the present study (Figure 2). The PPOS was not prepared or primed in advance with progestin, which was added in the second half of the stimulation cycle. Therefore, we purposely did not use the name PPOS and instead referred to this protocol as MS-LAP (mild ovarian stimulation with later administration of progestin). In this study, we analyzed the differences in clinical outcomes depending on the oral drug used. In particular, we compared the MS-LAP protocol using LTZ with that using CC with respect to the numbers of morphologically good blastocysts produced and the euploid rates.

In the present study, the backgrounds of the CC and LTZ groups were almost comparable with the exception of the AFC (the AFC in the CC group was significantly higher than that in the LTZ group, $p < 0.001$, Table 1). The number of retrieved, fertilized, and developed oocytes in the CC group was significantly higher than those in the LTZ group, but the number of blastocysts and morphologically good blastocysts were similar between the two groups (Table 2). The reason for the higher number of ICSI cases in the LTZ group rather than in the CC group was that the number of retrieved oocytes in the LTZ group was lower than that in the CC group, and the fertilization rate in our clinic was higher with ICSI than with conventional insemination. As a result, ICSI cases in the LTZ were higher than those in the CC group. Although the number of fertilized eggs was lower in the LTZ group, there was no difference in the number of blastocysts or morphologically good blastocysts between the two groups, therefore, we considered that the selection of fertilization method did not affect the euploidy rate. Indeed, the euploid rate in the LTZ group was 54.5%, however, and this was significantly higher than that in the CC group (38.0%, $p = .0231$) despite a similar TE biopsy rate. When considering only patients over 40 years of age (O40), the blastocyst formation rate in the LTZ-O40 group (58.5%) was significantly higher than that in the CC-O40 group (46.6%, $p = .028$). The euploid rate in the CC-O40 group (16.7%) was significantly lower than that in the LTZ-O40 group (40.5%, $p = .037$). We were unsure why this difference occurred when the only difference between the CC and LTZ groups was the oral medication used. We decided the difference must be due to the characteristics of the oral medications.

Letrozole (LTZ) which is one of the aromatase inhibitors. As such, it inhibits the aromatase enzyme and thereby reduces serum estradiol levels by blocking androgen conversion [25]. The use of LTZ in conjunction with gonadotropins has caused a decrease in estradiol levels in some studies, but this was neither beneficial nor harmful to oocyte number or to oocyte development. Studies that have followed the fertility preservation of patients have shown that adjuvant LTZ increases the numbers of both mature oocytes and cryopreserved embryos [26,27], but the abnormal fertilization rate was also increased by adjuvant LTZ [28]. A recent review reports that the number of retrieved oocytes, the oocyte quality, and the embryo development in an adjuvant LTZ group were comparable to those in a control group that had not used LTZ [29,30]. On the other hand, adjuvant LTZ could increase the number of retrieved oocytes and improve oocyte quality, without an improvement in embryo development [31,32]. These observations show both sides of adjuvant LTZ with gonadotropins for ovarian stimulation for and against. In these existing reports, the authors focused on the number of retrieved oocytes and oocyte and embryo quality. In the present study, however, we focused not only on the blastocyst formation rate but also on the euploid blastocyst rate. Although the previous studies compared adjuvant LTZ with a control, the present study evaluated the use of adjuvant LTZ with that of adjuvant CC. In this present study, the adjuvant LTZ group showed a higher euploid rate than that in the CC adjuvant group even though the LTZ group had a lower number of retrieved oocytes (Table 2). Moreover, this positive effect was confirmed even for the AMA women. Based on these results, there is no reason not to use LTZ together with mild ovarian stimulation.

Nevertheless, we were compelled to consider why LTZ adjuvant showed a higher euploid rate. Letrozole is well known to inhibit aromatase enzyme activity, which blocks the conversion of androgens into estrogens with a subsequent increase in intraovarian androgens [33]. At doses of 1 - 5 mg/day, letrozole inhibits aromatase activity by 97% - 99% [34]. An increase in intraovarian androgens has a fundamental trophic role in ovarian follicular development by augmenting the FSH receptor expression on granulosa cells [35]. Moreover, an interesting paper published by Weil and co-workers [36] reported that excess androgen induces an abundant primate ovary androgen receptor (AR) gene expression in the granulosa cells of healthy growing follicles where its expression is up-regulated by testosterone, and they reported a negative correlation between granulosa AR gene expression and programmed cell death [36]. In other words, those researchers inferred that an increase in androgens reduced the granulosa cell apoptosis and increased the number of euploid blastocysts.

Instead of using GnRH-agonist or GnRH-antagonist, to suppress the premature LH surge in ovarian hyperstimulation during ART treatment, the current trend is to use the progestin inhibitory effect [11,37,38]. Previous reports have speculated as to the mechanism of the progesterone inhibitory effect on LH surge [11,37,39]. The consensus has been that progesterone strongly inhibits pulsatile GnRH and LH secretion [39]. If this were true, however, an adequate number of well-developed follicles could not have been obtained via the use of the MS-LAP protocol in the present study. In the MS-LAP protocol, CC or letrozole that could work by means of endogenous gonadotropin secretion (both FSH and LH) could be effective for follicle growth. Therefore, we speculated that an inhibitory effect on LH surge by progesterone would not inhibit pulsatile GnRH and LH secretion. Currently, inhibition of the LH surge by progesterone has been identified in many species, and this includes humans [11,37,38]. Progesterone has been shown to block the LH surge by acting centrally to inhibit the surge of GnRH secreted by the hypothalamus [40]. Also, the current

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study indicates that the inhibitory effects that progesterone exerts on the LH surge are mediated by its receptor in the anteroventral periventricular nucleus (AVPV) [41]. Dynorphin, as a major inhibitory neuronal system and kisspeptin inhibitor, contributes significantly to LH surge secretion in rodents [42-44]. Reduced dynorphin levels are a prerequisite for LH surge secretion. The majority of dynorphin-containing neurons in the AVPV express both estrogen receptor and progesterone receptors [45]. When progesterone binds to its receptors in the AVAC following its administration while the dynorphin level remains high, an LH surge does not occur. On the other hand, pulsatile GnRH and LH secretion could be controlled by the kisspeptin in the arcuate nucleus (ARC) in the hypothalamus [46,47]. Therefore, progesterone administration could not inhibit pulsatile GnRH and LH secretion, and neither CC nor letrozole would work for ovarian stimulation.

Conclusion

In conclusion, integrating the aromatase inhibitor letrozole as part of an MOS protocol resulted in a higher euploid rate compared with that of a MOS that incorporated CC, although The TE biopsy rates for both were comparable. It is noteworthy that this positive effect was also seen in the AMA group. Moreover, the administration of progestin is started in the middle phase of ovarian stimulation to prevent LH surge, which reduces the amount of gonadotropin that using the PPOS method requires.

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Conflicts of interest

Koji Nakagawa, Takashi Horikawa, Keisuke Shiobara, Syunsuke Ishiyama, Hisayo Kataoka, Katsuki Nakao, Yuko Ojiro, Keiji Kuroda, Satoru Takamizawa, and Rikikazu Sugiyama declare no conflicts of interest that could appear to influence the results from this study.

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Authors contributions

Nakagawa K is the principal investigator. Horikawa T, Shiobara S, Ishiyama S, Kataoka S, Nakao K, Ojiro Y, Kuroda K, Takamizawa S, and Sugiyama S collaborated in the collection of the clinical data. Nakagawa K performed the statistical data analysis. Sugiyama R organized this study. All authors agree with the content of this manuscript. Everyone in the author list reviewed the final manuscript prior to submission.

Human rights statements and informed consent

This study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards, and it was reviewed and approved by the institutional review board of Sugiyama Clinic. All patients received and signed informed written consent forms before entering the study, and they also were given the option to withdraw from the study at any time during treatment.

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