**Title:** Drug-Free In Vitro Activation and Autologous Transplantation in Infertile Women with Diminished Ovarian Reserve: Pilot Study

**Running Title:** IVA in Infertile DOR women

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**Abstract**

Background and Objective: Poor ovarian response and diminished ovarian reserves significantly contribute to female infertility. Previous attempts have been made to enhance follicular growth and improve pregnancy outcomes in these patients. This study aimed to assess efficacy of the in vitro drug-free activation technique of the ovarian reservation and IVF stimulation cycle outcomes in DOR patients that referred to the Taleghani Infertility Center.

Materials and Methods: This pilot phase of a prospective cohort study investigated the impact of In Vitro Activation (IVA) on ovarian reservation and and outcome of IVF cycle in infertile women with diminished ovarian reserve at the Taleghani Infertility Center. Participants underwent general surgery and laparoscopy, involving the removal of a portion of one ovary, immediate transfer to the laboratory, dissection into small cubes, and subsequent re-implantation into the patient's ovary. Hormone levels, follicular growth, and pregnancy outcomes were recorded pre- and post-surgery.

Results: The study revealed a significant increase in Antral Follicle Count (AFC) from an average of 1.75 before IVA to 2.75 after IVA (P=0.033). Before IVA, the median E2 level was 93.5 (57.0), which reduced to 79.0 (35.0) after IVA, indicating a statistically significant difference. On average, 2.3 (0.8) oocytes were retrieved, among which 1.5 (0.7) were metaphase II oocytes. The observed pregnancy rate among the two patients was 22.2%.

Conclusion: The current study suggest that IVA may offer some favorable impacts on follicular growth and pregnancy outcomes among women with DOR .

Keywords:

In Vitro Activation , Autologous Transplantation, Infertile Women, DOR

**Introduction**

In recent years, there was in increase in number of infertile women with diminished ovarian reserve (DOR) (1). Various lifestyle risk factors, including smoking, alcohol consumption, poor diet, and specific medical treatments, significantly contribute to the observed upward trend of DOR (1). Responding poorly to standard ovulation stimulation treatments, these patients yield a limited number of low-quality antral follicles (2). Primary follicles in such cases exhibit difficulty in self-activation without external stimuli, hindering their growth to the primary stage and impeding the acquisition of mature eggs for in vitro fertilization (IVF) (3).

Numerous attempts to enhance follicular growth and improve pregnancy outcomes in these patients have been undertaken, but the outcomes have been disappointing. Traditionally, egg donation was the sole option, but emerging techniques aim to regenerate, rejuvenate, and activate ovarian germ cells. These methods encompass artificial gamete production from ovarian stem cells, intraovarian injection of activated platelet-rich plasma with calcium gluconate, autologous mitochondria transfer into oocytes, and androgen supplementation to heighten follicle sensitivity to external gonadotropin stimulation. However, routine implementation remains absent, and existing evidence is either weak or contentious (4). Primordial follicles in human ovaries face three fates: remaining dormant until needed, entering apoptosis directly from dormancy—resulting in the inability to produce normal mature eggs and early ovarian failure—or being selected for the growing follicle pool, thereby enhancing hormone secretion and ovulation. The balance between dormancy, death, and activation of these follicles is crucial for maintaining reproductive function (3).

Recent findings have indicated that drug-free ovarian cortex activation technology (drug-free IVA) can stimulate follicle growth in patients with primary ovarian failure and decreased ovarian reserve. This technique facilitates the activation of primary follicle pools in the resting state, potentially improving the likelihood of pregnancy (4). The current study aimed to investigate the efficacy of the in vitro drug-free activation technique of the ovarian reservation and IVF stimulation cycle outcomes in DOR patients that referred to the Taleghani Infertility Center in Tehran-Iran in 2022.

**Material and Methods**

Study design and study participants

The current study was the pilot phase of a prospective Cohort investigating the effects of IVA on the ovarian reservation and outcome of IVF cycle in infertile woman with diminished ovarian reserve referred to “Taleghani “infertility center. The inclusion criteria encompassed individuals under 40 years old, falling into group III or IV based on ovarian reserve reduction as per the POSEIDON criteria (5), with AMH levels below 1.2 ng/dl, FSH levels lower than 10 IU/L, and patients with a history of at least one failed stimulation cycle resulting in the inability to retrieve oocyte. Patients with male factor infertility, and positive history of hydrosalpinx or anatomical uterine disorders were excluded from the study Prior the surgery baseline characteristics of the study participants including age, body mass index (BMI), ovarian reserve status, and hormonal profile like Anti-Müllerian Hormone (AMH), Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), and Estradiol (E2) were recorded. Hormonal profile measurements were conducted on the second day of menses, while the count of pre-antral follicles was determined through sono-vaginal analysis following the IVA procedure. Three months later, another series of hormonal level assessments and antral follicle counts were performed. The associated data were recorded again during this phase.

Ethics approval

The study protocol underwent review and approval by the Ethics Committee and Review Board of Shahid Beheshti University of Medical Sciences. Before the surgery, the study protocol was thoroughly explained to the patients, and each participant completed an informed consent form. Patients were also allowed to withdraw the study at any phase.

Intervention

On the day of surgery, patients underwent general anesthesia. Through laparoscopy, a portion of ovarian tissue was extracted (each biopsy measuring approximately 5x5x3 mm). This tissue was promptly placed inside a sterile dish with media and transferred to the nearby laboratory. In the laboratory, within a sterile hood, the ovarian cortex was meticulously separated from the medulla under sterile conditions. A section of the cortex was forwarded to the pathology laboratory to ascertain the presence of any remaining follicles. Subsequently, the remaining cortex was promptly sliced into small cubes (measuring 1 x 1 x 1 cubic mm) using a scalpel blade. Approximately 15 to 20 of these pieces were carefully loaded into a transfer catheter containing minimal media and reintroduced into the ovarian incision.(6). After a period of 3 months, patients undergoing ovarian stimulation were administered the antagonist protocol using 150 units of Follicle-Stimulating Hormone (FSH). The protocol for the study participants involved daily subcutaneous injections of rFSH (150 IU/day), commencing on the second day of the menstrual cycle and continuing until the day of hCG administration. To prevent premature LH surges, they also received daily subcutaneous doses of 0.25 ganirelix, starting from Day 6 of stimulation and continuing up to the day of hCG administration, daily subcutaneous dose of 300 IU of hp-HMG, starting from Day 8 of stimulation and continuing until the day of hCG administration. If no follicles ≥11mm were observed on ultrasound between stimulation days 8-10, the cycle was terminated. Upon the ultrasound detection of at least two follicles reaching ≥18 mm, hCG administration at a dosage of 10,000 IU was initiated on the same day or the following day to prompt the final maturation of oocytes. Following a span of 34-36 hours post-hCG administration, patients underwent oocyte retrieval, and intracytoplasmic sperm injection (ICSI) procedures were performed. progesterone supplementation began on the day of oocyte retrieval. Actogest® 200 mg was administered daily intravaginally via suppository, while Progesterone Amp, 50mg/ml, was given intramuscularly on a daily basis. Embryo blast transfer took place on the 6th day post oocyte retrieval, with a maximum of two oocytes transferred per patient. The quality of the transferred embryos was evaluated using the Istanbul consensus workshop criteria, previously detailed by DeVos et al. Continuation of progesterone support persisted until the onset of menses or upon receiving a negative pregnancy test result. Patients underwent follow-up monitoring, during which hormone levels, follicular growth, and the overall ovarian response post-reimplantation were assessed. The associated data were recorded again during this phase. We also assessed follicular development and ovulation.

Outcome ascertainment

The study examined several outcomes, including the count of retrieved oocytes, number of oocytes reaching metaphase, quantity of embryos transferred, quality assessment of the transferred embryos, implantation rate, and occurrence of clinical pregnancy.

Statistical analysis

 We initially assessed the normality assumption using the Shapiro-Wilk test. Variables meeting the assumption were described using mean and standard deviation (SD), while non-normally distributed variables were presented using median and interquartile range (IQR). Dichotomous variables were described using frequency numbers and proportions. Paired t-tests or their non-parametric equivalent, the Wilcoxon test, were employed to assess pre- and post-IVA measurements. All statistical analyses were conducted using Stata software (Ver 17.0, College Station, Texas, USA). P-values less than 0.05 were considered significant

**Results**

The study participants had an average age of 36.8 years with a standard deviation of 3.2 years. The mean body mass index (BMI) was 25.1 with a standard deviation of 4.8. I, Seventy percent of patients presented with primary infertility, while the remaining cases exhibited secondary infertility after at least one spontaneous pregnancy. In all instances, the identified cause of infertility was attributed to female factors. The diagnosis of diminished ovarian reserve (DOR) was established according to the Poseidon criteria 3 and 4. The average duration of infertility recorded at 2.2 years with a standard deviation of 0.8 (Table 1).

In Table 2, the comparison of serum levels of menstrual hormones before and after IVA in the investigated patients was detailed. The average serum level of AMH was 0.58 (0.28) ng/dl before IVA, decreasing slightly to 0.56 (0.30) ng/dl after IVA, with no statistically significant difference observed (P=0.781). However, a significant increase in AFC was noted, with the average AFC rising from 1.75 before IVA to 2.75 after IVA (P=0.033). Before IVA, the median E2 level was 93.5 (57.0) pmol/L, which reduced to 79.0 (35.0) pmol/L after IVA, and this observed difference was statistically significant (P-value<0.05). No statistically significant change was observed in serum level of LH, and FSH prior and after IVA (P-value>0.05) (Table 2).

One cycle was canceled due to lack of response and lack of follicle growth on the 11th day of the cycle. among another 9 patients the average number of retrieved oocytes was 2.3 (0.8), which an average of 1.5 (0.7) were metaphase II oocytes. Embryo development was observed in 8 patients, while no embryos were formed for transfer in another patient, despite obtaining 1 metaphase II oocyte.The mean and standard deviation of the transferred embryos were 1.4±0.8. In total, 13 embryos were transferred during 8 patients that in three of them , single embryo and in the remaining two embryos were transferred , with BC quality showing the highest percentage among the transferred embryos. The proportion of embryos with B and C quality was 36.3% and 9.0%, respectively. The observed pregnancy rate across the two patients was 22.2%. Implementation rate was also provided as proportion of gestational sacs or fetal heartbeats observed during ultrasound (confirming a pregnancy) on the total number of embryos transferred and it was 27.3% (Table 2).

The first pregnancy was observed in a 38-year-old patient with a BMI of 32.4 kg/m². This patient experienced a 2-year duration of infertility due to secondary causes. The baseline levels of AMH, FSH, E2, and LH were 1.0, 10.4, 54.6, and 19.0, respectively. Post-intervention, these levels reached 1.1, 10.0, 68.0, and 18.0, respectively. Two oocytes were transferred, but only one proceeded to metaphasis, resulting in the development of a single embryo. The quality of the embryo was graded as BC. The second pregnancy occurred in a 36.1-year-old woman with a slightly elevated BMI of 27.1 kg/m². The duration of infertility was 3.0 years, attributed to a primary cause. At baseline, the levels of AMH, FSH, E2, and LH were 0.6, 12.1, 58.0, and 14.2, respectively. Following intervention, the hormone levels improved to 0.8 for AMH, 10.5 for FSH, 61.0 for E2, and 16.0 for LH. Two oocytes reached metaphase, resulting in the development of two embryos. The quality of the transferred embryos was graded as B and BC. One pregnancy, which involved twins progressed for 9 weeks, but unfortunately, despite yolk salk and fetal pole formation the heart did not form . Another case resulted in a 12-week missed abortion.

**Discussion**

Aging significantly diminishes the number of primordial follicles in the ovary, with a notable decline observed around the age of 40(7). Although dormant follicles persist in patients with Premature Ovarian Insufficiency (POI) and DOR, various attempts to regenerate, rejuvenate, or reactivate germ cells in the human ovary not have been successful (6).

Our findings indicated an increased average number of Antral Follicle Count (AFC) following IVA. Similar findings were reported by Kamawara et al (8)., demonstrating an increase in follicle growth among women diagnosed with DOR and POR following drug-free IVA procedures. According to Kamawara et al the observed growth in follicles and the increase in Antral Follicle Count (AFC) were attributed to the presence of residual secondary follicles and their rapid growth post IVA, as discussed (8). They refuted the role of AMH levels as a predictive factor in follicular growth post IVA (8). Our findings were consistent with these prior results, as we observed no significant change in the levels of AMH before and after IVA. Meanwhile, Hsueh et al. argued that drug-free IVA only activates secondary follicles and doesn't affect primordial follicles (9). They explained this by suggesting that during drug-free IVA, the Hippo signaling pathway is disrupted, impacting the early stages of follicle development (9). Another study by Ferreri et al. aimed to determine the clinical outcomes of utilizing the Drug-Free IVA technique for ovarian follicular activation. Their findings showed that this approach helped patients maintain their follicular waves for approximately 20 months post-surgery (2). Our study's pregnancy rate of 22.2% among patients with DOR who underwent the IVA procedure showcased a more favorable outcome compared to previous findings. Li et al. reported a lower pregnancy rate of 15.3% in DOR patients who underwent traditional IVF (10). In Huang et al.'s study on women with DOR, the observed pregnancy rate was 31.0%, slightly higher than our current study (11). However, it's noteworthy that our study involved older women compared to Huang et al (11). Various studies have highlighted the significant influence of maternal age on IVF-related pregnancy outcomes, with younger women demonstrating a higher likelihood of achieving clinical pregnancy and live birth post-IVF(6,11). While these findings lack confirmatory evidence due to the small sample size and absence of a comparison group, they serve as a potential steppingstone for further studies exploring the impact of drug-free IVA.

Our study marked one of the initial efforts to explore the clinical outcomes of drug-free IVA on follicular growth and pregnancy outcomes among women with DOR . Notably, our study was unique in its collection of data from 10 patients who underwent drug-free IVA, coupled with long-term patient follow-up to assess clinical outcomes, including chemical and clinical and ongoing pregnancies. However, it's essential to interpret our findings in light of certain limitations. The primary limitations of our study were the small sample size and the absence of a comparison group. Future clinical trials with randomization, larger sample sizes, and extended follow-up periods may offer deeper insights into both the benefits and potential adverse effects of drug-free IVA among women with DOR .

**Conclusion**

In conclusion, the findings suggest that IVA may offer some favorable impacts on follicular growth and IVF outcomes among women with DOR, demonstrating a slight increase in AFC and pregnancy rates. Nonetheless, further trials involving larger sample sizes and comparison groups are essential to substantiate these findings and establish a clearer understanding in this regard.

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**Conflict of interests**

The authors declare that they have no competing interests.

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Table 1: Study participants baseline characteristics

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| --- | --- |
| Characteristics  | N=10  |
| Age (Year), Mean (SD) | 36.8 (3.2) |
| BMI (kg/m2), Mean (SD) | 25.1 (4.8) |
| Cause of infertility, n (%)  |  |
| Primary  | 7 (70.0%) |
| Secondary | 30 (30.0%) |
| Duration of infertility (Year), Mean (SD) | 2.2 (0.8) |

Table 2: Follicular growth and serum hormone before and after drug-free IVA in patients with DOR and POI

|  |  |  |  |
| --- | --- | --- | --- |
| Hormone  | Before IVA | After IVA | P-value |
| AMH, Mean (SD) | 0.58 (0.28) | 0.56 (0.30) | 0.781 |
| AFC, Mean (SD) | 1.75 (0.70) | 2.75 (0.70) | 0.033 |
| E2, Median (IQR) | 93.5 (57.0) | 79.0 (35.0) | 0.002 |
| LH, Mean (SD) | 13.41 (3.48) | 12.07 (4.0) | 0.083 |
| FSH, Mean (SD) | 12.71 (3.60) | 12.66 (3.91) | 0.637 |

Table 3: Pregnancy outcomes of the patients who underwent drug free in-vitro activation (IVA)

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| --- | --- |
| Characteristics  | N=9  |
| N of oocytes, Mean (SD)  | 2.3 (0.8) |
| N of metaphase II oocytes, Mean (SD) | 1.5 (0.7) |
| N of embryo transferred, Mean (SD) | 1.4 (0.8) |
| Quality of transferred embryos, n (%) |  |
| B | 4 (36.3%) |
| BC | 7 (63.6%) |
| C | 1 (9.0%) |
| Implantation rate  |  |
| N of gestational sacs/ n of embryo transferred  | 3/11 (27.3%) |
|  |  |
| Pregnancy outcome  |  |
| Yes | 2 (22.2%) |
| No | 7 (77.8%) |