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Unlocking Dormant Ovarian Function: A Phase I Clinical Trial of Regenerative Stem Cell Therapy Combined with in Vitro Ovarian Activation

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Abstract

Premature ovarian failure (POF) is a condition that causes early decline of ovarian function and infertility in women under 40, with limited treatment options that can effectively restore ovarian activity. In this Phase I clinical trial, the safety and initial effectiveness of combining in vitro ovarian activation (IVA) with mesenchymal stem cells derived from Wharton's jelly (WJ-MSCs) were evaluated in ten women diagnosed with POF. Each participant received unilateral IVA followed by local injection of WJ-MSC and was followed up over a twelve-month period. The treatment was well tolerated, with no adverse events reported. Among the eight participants who completed the follow-up, six regained regular menstrual cycles and showed a notable decrease in serum FSH levels. Additional improvements included reduced menopausal symptoms and increased endometrial thickness, though changes in anti-Mullerian hormone and estradiol levels were not statistically meaningful. These results indicate that combining WJ-MSC therapy with IVA may support ovarian tissue and enhance residual follicular activity. Further studies with larger, controlled cohorts are necessary to validate these findings and refine regenerative treatment strategies for women with POF.

Introduction

Premature ovarian failure is a challenging and often irreversible condition that profoundly affects young women under the age of 40, compromising not only their reproductive potential but also their sense of femininity and maternal identity. This condition is defined by the presence of amenorrhea, reduced circulating estrogen concentrations, impaired fertility, elevated serum gonadotropin levels, and the absence of mature ovarian follicles (1). Despite considerable understanding of the etiological factors associated with this condition including genetic abnormalities, autoimmune disorders, medical interventions such as chemotherapy or radiotherapy, and lifestyle factors such as cigarette smoking and excessive alcohol consumption there is currently no established definitive treatment (2, 3). Standard treatments, including hormone replacement therapy (HRT) and assisted reproductive technologies (ART), demonstrate limited efficacy, particularly with respect to restoring ovarian function and achieving natural pregnancy. When standard medical treatments like HRT or fertility procedures fall short of helping someone conceive, it's a difficult reality to face. For many women, the next step using donated eggs or embryos feels like a logical clinical solution, but in practice, it's far more complicated.

Most people don't realize how heavy this decision weighs on the parents-to-be. Beyond the high costs and the logistical stress, there is a deep, often unspoken grief that comes with the realization that they won't share a genetic connection with their child. It's a very personal hurdle that many people just aren't ready to clear (4). But there's another side to this that often gets overlooked: the donors themselves. Going through an egg donation cycle is physically and emotionally taxing, and studies are starting to show that it can take a real toll on the donors, sometimes even leading to struggles with depression. It's a reminder that these fertility choices aren't just about the parents; they involve complex, human experiences for everyone stepping into the process (5, 6). Although oocyte or embryo donation is often considered the most efficient clinical recourse for ovarian insufficiency, these approaches do not

address the underlying pathology. Given the presence of a fibrotic ovarian microenvironment and dormant follicles which typically fail to respond to conventional hormonal therapies like HRT a paradigm shifts toward restoring autologous ovarian function is imperative. Current interventions often fall short by focusing solely on systemic hormonal replacement rather than targeting the local ovarian tissue. By synthesizing recent advancements in regenerative medicine, there is significant potential to develop more effective, targeted protocols aimed at mitigating ovarian fibrosis and activating dormant follicles to achieve a more favorable reproductive outcome.

Based on previous studies, fibroblast proliferation and extracellular matrix (ECM) deposition cause ovarian fibrosis, which is usually associated with the progression of POF. As reported in several studies, mesenchymal stem cells (MSCs) have attracted considerable attention as a therapeutic approach for addressing ovarian tissue fibrosis due to their multipotent characteristics, immunomodulatory properties, and potential to enhance tissue repair and regeneration (7, 8). MSCs have shown a special anti-fibrotic effect by regulating cytokines related to fibrosis such as matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), transforming growth factor β1 (TGF-β1), and endothelin-1 (ET-1). In addition, anti-inflammatory and immunomodulatory effects may be other mechanisms by which MSCs restore ovarian function. MSC transplantation can regulate anti-inflammatory regulatory T cells (Treg) and inhibit pro-inflammatory Th_{17} cells (9). Studies have demonstrated that transplantation of UCMSCs into the ovaries of chemotherapy-induced premature ovarian insufficiency (POI) mouse models leads to the restoration of ovarian endocrine and exocrine functions, improvement of fertility potential, and successful pregnancies. These therapeutic effects are associated with decreased serum follicle-stimulating hormone (FSH) levels, increased anti-Müllerian hormone (AMH) concentrations, resumption of ovulation, and enhanced ovarian angiogenesis, which promotes granulosa cell proliferation and follicular growth. Among UCMSCs, Wharton's jelly–derived mesenchymal stem cells represent a particularly promising cell source due to their high proliferative capacity, low immunogenicity without the need for post-transplant immunosuppression, lack of tumorigenicity, ease of noninvasive collection from the umbilical cord, and ethical advantages (10–12). However, an important question remains as to whether stem cell transplantation alone is sufficient to induce ovarian regeneration in premature ovarian failure (POF). It appears that, beyond stem cell transplantation alone, an additional stimulatory intervention may be required to reactivate long-dormant ovaries in patients with premature ovarian failure. As is widely recognized, combination therapeutic strategies in medicine often lead to superior clinical outcomes. In this context, integrating stem cell–based therapy with IVA may constitute a more effective approach for restoring ovarian function. Indeed, as reported in the literature, IVA has been employed as a strategy to induce the reactivation of ovarian folliculogenesis (13, 14). The procedure of IVA begins with the retrieval of a small piece of ovarian tissue from the patient. Once the follicles have been stimulated, the tissue is re-implanted into the ovarian cortex of the patient's body. This technique promotes the activation of dormant ovarian follicles by disrupting the Hippo signaling pathway and modulating the PTEN/PI3K/AKT/FOXO3 signaling cascade, which stimulates the growth of primordial follicles (15, 16). Several studies have reported successful pregnancies and live births

following the application of in vitro activation, demonstrating its potential as a fertility treatment (16–20).

Given the complementary mechanisms of IVA and WJ-MSC transplantation, we hypothesized that their combined application could synergistically improve ovarian function and clinical outcomes in women with premature ovarian failure (POF). Accordingly, we conducted a phase I clinical trial to evaluate the safety, feasibility, and preliminary clinical efficacy of this novel therapeutic approach. We anticipate that these findings will inform future studies and contribute to the development of more effective treatment strategies for POF.

Invitro activation (IVA)

Experimental studies have demonstrated that mechanical disruption of the ovarian cortex, including tissue fragmentation, alters cytoskeletal organization by promoting actin polymerization. This process suppresses Hippo pathway activity and facilitates the nuclear translocation of Yes-associated protein (YAP), a key downstream effector of the pathway. Within the nucleus, YAP interacts with TEAD transcription factors, resulting in increased expression of genes involved in cell proliferation, survival, and tissue regeneration, including cysteine-rich angiogenic inducer 61 (CYR61/CCN1) and members of the baculoviral inhibitor of apoptosis repeat-containing (BIRC) family. Collectively, these molecular events create a favorable microenvironment that supports follicle activation and subsequent growth (18, 21). Based on this mechanistic rationale, we applied controlled mechanical stress to the ovarian cortex using a laparoscopic chopping technique designed to reproduce the biological effects of ovarian fragmentation while preserving overall ovarian architecture. This minimally invasive approach was intended to induce localized tissue remodeling and stimulate Hippo pathway modulation without extensive tissue excision. The intervention was conceptually derived from the principles of IVA, in which ovarian fragmentation, together with activation of the PI3K/Akt signaling pathway by growth factor stimulation, has been employed to promote the activation of residual primordial follicles in women with premature ovarian insufficiency. Figure 1 illustrates the proposed mechanism by which laparoscopic mechanical stimulation may enhance follicular activation through Hippo pathway disruption and subsequent activation of downstream proliferative and anti-apoptotic signaling pathways.

Figure 2

Evidence from both experimental animal models and clinical studies suggests that in vitro activation can stimulate the growth of residual follicles and partially restore ovarian function. This approach combines ovarian tissue fragmentation with activation of the PI3K/AKT signaling pathway, followed by auto-transplantation of the treated tissue, thereby promoting follicular activation in selected patients (13, 22, 23).

In a clinical investigation involving 27 women with POF, IVA induced follicular growth in eight patients, enabled the retrieval of mature oocytes in five cases, and resulted in one live birth, highlighting its

potential as a fertility-preserving strategy for women with residual ovarian reserve. Despite these encouraging outcomes, the overall clinical efficacy of IVA remains limited. Age-related deterioration in oocyte quality and the inability of IVA alone to reverse the pathological alterations within the ovarian microenvironment may restrict its therapeutic benefit (24).

Stem cell therapy

To overcome these limitations, combining IVA with regenerative therapies has emerged as a promising strategy. Mesenchymal stem cells (MSCs) have attracted considerable attention because of their ability to restore ovarian function primarily through paracrine mechanisms rather than direct differentiation. The diverse array of bioactive molecules secreted by MSCs including growth factors, cytokines, chemokines, and extracellular vesicles can suppress apoptosis, attenuate fibrosis and inflammation, stimulate angiogenesis, and protect granulosa cells, thereby creating a microenvironment more conducive to follicular survival and development (14, 16). Consequently, the integration of IVA with MSC therapy may provide complementary therapeutic effects by simultaneously activating dormant follicles and improving the structural and functional integrity of ovarian tissue.

Although most preclinical and clinical studies investigating MSC therapy have focused on ovarian damage induced by chemotherapy, radiotherapy, or bone marrow transplantation, particularly in rodent models, early clinical evidence in women with POF has also shown encouraging results (25). Nevertheless, additional well-designed clinical trials with larger patient populations and longer follow-up periods are needed to determine the long-term efficacy, optimal treatment protocols, and safety of combining MSC transplantation with IVA for ovarian regeneration (26).

A growing body of evidence has highlighted the therapeutic potential of mesenchymal stem cells (MSCs) derived from different tissue sources for the treatment of premature ovarian failure (POF) (27–29).

Bone marrow-derived stem cells (BMSCs) have been reported to improve ovarian endocrine and reproductive function by elevating estrogen levels, reducing FSH concentrations, increasing follicular density, and preventing granulosa cell apoptosis, largely through vascular endothelial growth factor (VEGF)-mediated pathways and other gene regulatory mechanisms (30). Similarly, adipose-derived stem cells (ADSCs) contribute to ovarian repair by enhancing angiogenesis, supporting follicular regeneration, and alleviating structural tissue damage within the ovary (31). Umbilical cord-derived MSCs (UC-MSCs) have also demonstrated beneficial effects, particularly in reducing ovarian fibrosis through modulation of the TGF-β/Smad3 signaling pathway (31). In addition, amniotic-derived stem cells exhibit anti-inflammatory and regenerative properties, including suppression of pro-inflammatory cytokines and promotion of follicular recovery (32).

Beyond the general effects of MSCs, emerging evidence suggests that their therapeutic efficacy can be further enhanced through molecular modification. In this context, heme oxygenase-1 (HO-1) has been identified as a key regulator that significantly improves the regenerative capacity of UCMSC therapy in POF. Experimental studies in animal models have shown that HO-1–overexpressing UCMSCs markedly

restore ovarian function by promoting granulosa cell survival, reducing apoptosis, improving endocrine profiles characterized by increased estradiol (E2) and AMH levels, and decreasing FSH and luteinizing hormone (LH) concentrations. These changes are accompanied by reduced ovarian fibrosis and an increased number of morphologically healthy follicles. In contrast, UCMSCs lacking HO-1 exhibit significantly diminished therapeutic efficacy. Mechanistically, HO-1 enhances ovarian repair by activating the JNK/Bcl-2 signaling axis, thereby promoting autophagy in granulosa cells, as evidenced by increased LC3-II, Beclin1, and Atg5 expression, decreased p62 levels, and enhanced autophagosome formation. Pharmacological modulation using JNK activators and inhibitors has further confirmed the essential role of this pathway in mediating HO-1–driven effects. In addition, HO-1 contributes to immune modulation by increasing circulating $CD8^{+}CD28^{-}$ regulatory T cells and elevating anti-inflammatory cytokine IL-10 levels, thereby establishing a more favorable immunological environment for ovarian regeneration. Collectively, these findings suggest that HO-1 represents a promising molecular target for optimizing stem cell–based therapies in the management of POF (33).

Materials and methods

Study design

This study was registered in the Iranian Registry of Clinical Trials (IRCT) on November 14, 2021, with the number IRCT20211021052828N1 (available online at https://irct.ir/trial/59564). This phase I uncontrolled clinical trial study was designed to assess the safety and primary efficacy of the IVA technique along with WJ-MSC injection in patients with POF. After obtaining ethical approval (IR.GUMS.REC.1400.325) from Guilan University of Medical Sciences, the written informed consent was signed by all the study participants. Ten out of eighteen POF patients between the ages of 18–40 Y/O referred to Mehr Infertility Institute, Rasht during November 2021- August 2022 were included in the study based on the inclusion criteria as follows: normal karyotype XX46, presence of both ovaries, normal uterus anatomy and normal serum thyroid-stimulating hormone (TSH) level, secondary amenorrhea for at least 6-months, serum AMH level under 0.42 ng/mL as an indicator of diminished ovarian reserve, FSH levels upper 25 IU/L on two occasions at least four to six weeks apart, and/or previous Assisted Reproductive Technology (ART) failure due to limited ovarian response (poor responder) and also, exclusion criteria included thyroid dysfunction (hypothyroidism, hyperthyroidism), history of immunological disorders, type 1 or 2 diabetes, heart diseases, liver disease, kidney diseases, extensive endometriosis, breastfeeding, reproductive or breast cancer, any severe mental disorder, smoking over 35 years old, uncontrolled high blood pressure and irregular use of hormonal drugs. Table 1 shows the general characteristics of patients diagnosed as having POF and selected.

Table 1
The participants' demographic and clinical characteristics

Patients	1	2	3	4	5	6	7	8
Age (y)	37	34	29	33	40	30	32	34
Karyotype	N	N	N	N	N	N	N	N
Amenorrhea for more than 6-months	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
AFC (Number)	4	No	No	2	No	No	No	No
Dominant follicle size	< 10	No	No	< 10	No	No	No	No
Endometrial thickness	Thin	Thin	Thin	Thin	Thin	Thin	Thin	Thin
E2 (pmol/L)	18	19.6	32	123	15	41	52	84
LH (IU/L)	33	43.9	51.1	32	99	20	41.5	10.4
The average of two FSH measurements (IU/L)	79	47	52.7	25	128	68	94.3	26
AMH (ng/mL)	0.01	0.01	0.02	0.02	0.03	0.02	0.05	0.05
Symptoms of menopause	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

AFC, antral follicle count; *AMH*, anti-Müllerian hormone; *E2*, Estradiol; *FSH*, follicle stimulating hormone; *LH*, luteinizing hormone; *N*, normal.

Table 1

The left ovary of the eligible patients was subjected to ovarian cortex IVA accompanied by WJ-MSC injection and the other was considered as a sham control at the injection site. The follow-up program was designed to assess safety and some efficacy issues for twelve-months after the treatment.

WJ-derived MSC isolation and expansion

Umbilical cord tissue was obtained following written informed consent from eligible mothers in accordance with local ethical guidelines. Donors were screened and confirmed negative for human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotropic virus (HTLV), Epstein–Barr virus (EBV), Toxoplasma gondii, and rapid plasma reagin (RPR) testing for cesarean deliveries. Isolation and ex vivo expansion of WJ-MSCs were conducted under Good Manufacturing Practice (GMP) conditions. Briefly, umbilical cord specimens were rinsed three times with Dulbecco's phosphate-buffered saline (DPBS). After removal of the umbilical vessels, the Wharton's jelly was dissected into approximately 2–3 mm fragments under sterile conditions. The tissue fragments were digested with 0.5 U/mL type IV collagenase for 1 hour at 37°C. Following enzymatic digestion, the cell suspension was centrifuged at 400 × g for 5 minutes, and the resulting pellet was resuspended in

Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cultures were maintained under standard conditions, with medium replacement performed twice weekly. Cells at passage 3 were characterized by flow cytometry to confirm the mesenchymal stromal cell phenotype through assessment of the positive markers CD105, CD73, and CD90 and the negative hematopoietic markers CD45 and CD34. Before clinical release, comprehensive quality control testing was performed, including sterility assessment (Merck, Germany), mycoplasma detection (Payeshgene, Iran), endotoxin testing (Bioendo, China), Gram staining (Baharafshan, Iran), karyotype analysis, and cell viability evaluation (Merck, Germany), in accordance with established GMP quality standards (10).

IVA and cell transplantation

All patients were subjected to the IVA technique. Briefly, a 1×1 cm piece of left ovarian cortex was removed by laparoscopy. The harvested tissue was fragmented into small pieces of about 1×1 mm, which were transplanted via a catheter into the corresponding ovary and sutured in place (Fig. 3a-e).

Stem cell injection into the ovary

At their third passage, 10×10^6 WJ-MSCs were co-transplanted alongside the fragmented tissues into the patient's left ovary, by a gynecological surgeon through laparoscopy (Fig. 3f). The right ovary served as the control for safety evaluation.

Figure 3

Patient follow-up

The patients were followed up for up to 12-months after the transplantation for safety assessment, primary and secondary outcomes. The primary objective was to evaluate the safety of the therapy. The patients were monitored for the occurrence of any adverse side effects related to MSC treatment including fever, adverse reactions at the injection site (bleeding, swelling, pain, itching, and infection), constipation, fatigue, insomnia, diarrhea, allergy/anaphylaxis, skin reactions such as itching and hives, reddened and pale skin, low blood pressure, difficulty in breathing, suffocation, arrhythmia (paroxysmal supraventricular tachycardia, ventricular tachycardia, atrial fibrillation, ventricular fibrillation), urticarial/dermatitis, central nervous system disorders (dizziness and headache), nausea, vomiting, seizures, anemia, vascular disorders (vascular thrombosis including venous and arterial thrombosis and vasculitis), metabolic and nutritional disorders (malnutrition), infection, graft-versus-host disease (GvHD), tumor and death.

The primary outcomes were to monitor patients for potential side effects at specific intervals immediately post-treatment, within 24 hours, one week, two weeks, one-month, three-months, six-months, and twelve-months and to assess the resumption of menstruation throughout a 12-months follow-up period. The secondary outcomes were to measure and compare the serum level of AMH, FSH,

and E2 serum level, and also examination of endometrial thickness in the third and sixth month after the treatment; to control menopause symptoms (reduction of hot flashes, increase of sexual desire, and improvement of sleep) up to 12-months after the treatment.

Statistical analysis

Analysis of data was performed using IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, NY, USA). All data were presented as mean ± standard deviation. Kolmogorov-Smirnov test was used to test the normality of the data. The repeated measures were analyzed by one-way ANOVA. In the present study, $P > 0.05$ was considered statistically significant.

Results

Patients' recruitment

In this study, the recruitment of patients with POF started in 2021/12 and patient follow-up continued until December 2023. In total, ten patients participated in the present study based on the inclusion and exclusion criteria, of which two patients refused to continue the treatment. Table 1 summarizes the demographic and clinical characteristics of the participants.

Primary outcomes

Safety assessment

The treatment exhibited an excellent safety profile and was well tolerated by all participants. No treatment-related adverse events or clinically significant side effects were identified during the intervention or throughout the one-year follow-up period. Furthermore, continuous clinical monitoring revealed no delayed complications or unexpected safety concerns, indicating that the intervention remained safe over both the short- and long-term observation periods.

It should be noted that, although the results presented in the graphs were standardized to cover a six-month follow-up period, the assessment of long-term adverse effects, along with specific outcomes such as the return of menstruation and the alleviation of menopausal symptoms, was continued for up to one year.

Menstruation return

Menstrual function was evaluated through monthly follow-up assessments to determine the restoration and maintenance of regular menstrual cycles after treatment. Among the eight patients included in the study, six demonstrated sustained regular menstruations throughout the 12-month follow-up period, representing a statistically significant improvement compared with baseline ($P = 0.002$) (Fig. 4). These findings suggest that the intervention may have contributed to the recovery and long-term maintenance of normal menstrual function. During the follow-up period, four patients subsequently underwent in vitro

fertilization (IVF) as part of assisted reproductive treatment. However, reproductive outcomes, including pregnancy and live birth rates, were not assessed or reported, as the primary objective of the study was to evaluate the safety and clinical efficacy of the intervention rather than fertility outcomes.

Figure 4

Secondary outcomes

The serum level of AMH

The effect of time on the average AMH values was investigated using the Greenhouse-Geisser test, due to the default of sphericity. Comparison of the serum AMH with LSD method showed that there is no significant difference between the given time points before and 3- and 6-months after the treatment, (P = 0.217), (Fig. 5).

Figure 5

The serum level of FSH

The effect of time on the average values of FSH was investigated using the Assumed Sphericity test, due to the assumption of sphericity. The difference between the serum level of FSH at different time points was not significant (P = 0.119). Comparing the averages of different periods with LSD method showed that there is a significant difference between the FSH level before and third month after the treatment (P = 0.024), while there is no significant difference between the level of FSH before and six-months after the treatment (P = 0.346) (Fig. 6).

Figure 6

The serum level of LH

In this study, the changes in the serum level of LH were analyzed using the Assumed Sphericity test, due to the assumption of sphericity. The difference between the averages of LH serum levels at different time points was significant (P = 0.013). Comparing the averages of different time points with the LSD method showed that there is a significant difference between the baseline point and three and six-months after the treatment (Fig. 7).

Figure 7

The serum level of E2

E2 hormone was evaluated using the Assumed Sphericity test, and the difference between the averages at different time points was significant (P = 0.119). The results of comparing the averages of different time points of E2 level with the LSD method showed that there is no significant difference between before and third and sixth-months after the treatment (Fig. 8).

Figure 8

Menopause symptoms

Menopausal symptoms, including hot flashes, insomnia, mood disturbances, vaginal dryness, and decreased libido, were evaluated throughout the follow-up period. A significant reduction in the overall severity of these symptoms was observed in six patients and was maintained throughout the 6-month follow-up period (P = 0.031) (Fig. 9). These findings suggest a sustained improvement in menopausal symptoms following treatment. As noted previously, symptom assessment continued for one year after the intervention. However, to ensure consistency in data presentation and facilitate comparison across all outcome measures, only the results from the initial six-month were included in the graphical analysis.

Figure 9

Endometrial thickness

Endometrial thickness was evaluated by transvaginal ultrasonography at baseline and at 3- and 6-months following treatment. Statistical analysis demonstrated a significant increase in mean endometrial thickness across the three assessment time points (P = 0.007) (Fig. 10). The observed increase in endometrial thickness is indicative of improved endometrial regeneration and is consistent with the restoration of ovarian hormonal activity, as reflected by the recovery of regular menstrual cycles. These findings suggest that the intervention may have contributed to the functional recovery of the endometrium and the normalization of reproductive endocrine function.

Figure 10

Discussion

Premature ovarian failure is characterized by a progressive decline in ovarian function resulting from depletion of the primordial follicle pool, accelerated follicular atresia, impaired communication between oocytes and granulosa cells, and extensive ovarian fibrosis (2, 34).

The therapeutic strategy evaluated in the present study was developed based on accumulating evidence that mechanical stimulation or IVA of ovarian tissue can reactivate remaining dormant follicles through modulation of the Hippo signaling pathway. Kawamura et al. were among the first to report that IVA, when combined with ovarian tissue reimplantation, could induce follicular growth and result in successful pregnancies in women with primary ovarian insufficiency (16).

Nevertheless, activation of dormant follicles within a fibrotic ovary may not, by itself, be sufficient to achieve meaningful functional recovery. Given the multifactorial nature of premature ovarian failure, IVA is likely to require combination with additional regenerative interventions that target other aspects of ovarian pathology. Among these, granulosa cell dysfunction is recognized as a critical determinant of disease progression because of its essential role in maintaining follicular development and ovarian

homeostasis. Granulosa cells provide metabolic and structural support to the developing oocyte, regulate steroid hormone biosynthesis, and mediate bidirectional signaling that is essential for normal folliculogenesis. Disruption of this functional interaction promotes granulosa cell apoptosis, leading to follicular degeneration, impaired estrogen production, and progressive loss of ovarian reserve. In addition, excessive extracellular matrix deposition and ovarian fibrosis further compromise the ovarian microenvironment by reducing vascularization, limiting nutrient delivery, and diminishing the survival and developmental potential of residual follicle (34).

Taken together, the combination of cell-based therapy with in vitro activation may represent a synergistic strategy for the management of premature ovarian failure, as it simultaneously aims to activate dormant follicles while improving the ovarian microenvironment to support follicular survival and growth. Although the precise mechanisms underlying stem cell–mediated ovarian restoration remain incompletely understood, accumulating evidence suggests that paracrine signaling from transplanted cells may contribute to follicular activation and tissue repair. In particular, stem cells and their secreted bioactive factors may support processes related to neo-oogenesis, potentially leading to the formation of new follicle-like structures in the adult ovary. This regenerative process has been hypothesized to involve activation and differentiation of bipotent ovarian stem cell populations located within the tunica albuginea, which may give rise to granulosa-like cells and germ cell precursors capable of contributing to primordial follicle assembly (28, 39–42).

Based on the therapeutic rationale described above, the intervention also yielded promising preliminary observations suggestive of potential clinical benefit, which are discussed in the following sections. Nevertheless, because this study was conducted as a phase I clinical trial, its primary objective was to establish the safety and feasibility of the combined therapeutic approach rather than to evaluate treatment efficacy. Therefore, the most important outcome of the present study was the demonstration of a favorable safety profile. No instances of graft-versus-host disease, tumorigenesis, severe immune-related adverse events, or other serious treatment-associated complications were observed during the intervention or throughout the one-year follow-up period. These results further support the clinical safety of combining WJ-MSCs with the IVA method and reinforce the potential of this strategy as a regenerative cell-based therapy. The absence of clinically significant immune complications is consistent with the low immunogenicity of WJ-MSCs, which is largely attributed to their limited expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory surface markers. In addition, the well-established immunomodulatory and anti-inflammatory properties of WJ-MSCs may contribute to their excellent tolerability by regulating immune responses and creating a favorable tissue microenvironment. Collectively, these characteristics support the feasibility of repeated or allogeneic administration of WJ-MSCs and further strengthen their therapeutic potential for regenerative medicine applications.

Alongside the favorable safety profile, the intervention was associated with promising endocrine changes during the six-month observation period, suggesting a potential therapeutic effect on ovarian function.

Among endocrine markers, FSH remains one of the most reliable clinical indicators of ovarian responsiveness and is widely recommended by ESHRE guidelines for the evaluation of ovarian insufficiency (38).

Given the fluctuating nature of gonadotropin secretion in amenorrheic patients, an FSH cutoff value of > 25 IU/L was used in this study in accordance with established criteria. In participants who resumed menstruation, FSH measurements were standardized to the early follicular phase (days 2–3 of the cycle) to ensure consistency. Patients who resumed menstrual function demonstrated a progressive decline in serum FSH levels over the six-month follow-up period. Patients who resumed menstrual function showed a gradual decrease in serum FSH levels during the 6-month follow-up period. Although this downward trend was not significant in all participants at the final assessment point, it was due to the effect of a single patient's test result, which had a significant increase in FSH at the 6-month test, which in turn affected the statistical result at the group level. This highlights the significant impact of interindividual variability in studies with limited sample sizes. Furthermore, these findings emphasize the importance of appropriate patient selection, especially with regard to disease duration and specific POF subtype, as these variables may play an important role in determining treatment response. Consequently, future research with more stringent inclusion criteria and larger study populations is needed to more accurately identify patients who are most likely to benefit from this treatment strategy.

Reproductive function in women is tightly regulated by circulating sex steroid hormones. Estradiol, produced by both theca and granulosa cells within developing follicles, plays a central role in the hypothalamic–pituitary–ovarian axis by exerting negative feedback on the pituitary gland, thereby regulating FSH secretion (28). Although no statistically significant differences in E2 levels were detected across baseline, 3-month, and 6-month evaluations, early improvements in hormonal profiles were observed at the 3-month follow-up after treatment. This transient endocrine response may partly explain the significant reduction in serum FSH recorded during the early post-treatment period. The absence of sustained statistically significant changes over the full follow-up is likely related, at least in part, to the limited sample size and consequent reduction in statistical power. Nevertheless, the overall hormonal trends indicate a possible biological effect of the intervention and highlight the need for confirmation in larger, well-powered studies with extended observation periods. Although no meaningful reduction in FSH levels was observed, LH concentrations demonstrated a significant decline from baseline that persisted throughout the six-month follow-up period. As primary gonadotropins produced by the anterior pituitary, FSH and LH are critical regulators of folliculogenesis and oocyte maturation, and their secretion is governed by estradiol-mediated negative feedback mechanisms. In this regard, a reduction in LH is frequently considered an early indicator of improved endocrine responsiveness. Accordingly, the sustained decrease in LH observed in the present study may reflect an initial stage of partial hormonal recovery, even in the absence of a corresponding rise in estradiol levels. It is conceivable that further optimization of the therapeutic regimen, including adjustments in cell dose or administration frequency, could yield additional endocrine improvements, such as subsequent decreases in FSH and increased estradiol production. Overall, these findings suggest that Wharton’s jelly-derived mesenchymal stem cell (WJ-MSC) transplantation may contribute to partial re-establishment of hormonal homeostasis in

patients with POF/POI. In line with these results, comparable endocrine improvements have been reported in experimental studies, including a rat model of POF treated with WJ-MSCs, where normalization of sex hormone profiles was observed (43).

With respect to ovarian reserve, no statistically significant change in serum AMH levels was observed following treatment. However, a modest increase in AMH was detected in a subset of participants, which may reflect localized activation of residual follicles accompanied by granulosa cell proliferation and differentiation. This interpretation is supported by the ultrasonographic observation of follicular growth in both ovaries, including the treated ovary, suggesting that the intervention may have stimulated residual follicular activity (35, 36). Given that AMH is primarily produced by granulosa cells of preantral and small antral follicles and correlates closely with the AFC, even a slight increase in circulating AMH may be indicative of enhanced activity within the remaining follicular pool, although not necessarily of a substantial recovery of ovarian reserve (37). However, AMH has inherent limitations in accurately reflecting meaningful recovery of ovarian function in premature ovarian failure, as they are primarily used as general indicators of ovarian reserve rather than definitive markers of disease resolution. Nevertheless, its circulating levels are highly dependent on the size of the remaining follicular pool. Consequently, individuals with severely diminished ovarian reserve typically exhibit low AMH concentrations, and minor fluctuations may not necessarily indicate true improvements in ovulatory function or oocyte quality [28].

An encouraging clinical finding was the recovery of regular menstrual cycles in six patients, and this response was sustained for the duration of the one-year follow-up. Overall, six out of eight patients maintained regular menstruation during the follow-up period, and improvements in menopausal symptoms were also reported. At 12-month follow-up, four patients proceeded to in vitro fertilization (IVF) cycles for assisted reproduction. Menopausal symptom relief is another clinically relevant outcome in patients with POF. Although various hormonal and non-hormonal therapies exist, their effects are typically transient and require continuous administration. In the present study, patients experienced improvement in menopausal symptoms for up to one-year following a single combined intervention of IVA and WJ-MSC therapy. In addition, endometrial thickness increased and approached values closer to physiological norms, suggesting partial restoration of endometrial responsiveness.

The clinical findings of the present study are consistent with previous experimental and early clinical evidence supporting the regenerative potential of mesenchymal stem cells (MSCs) in ovarian insufficiency. MSCs derived from bone marrow, adipose tissue, and umbilical cord have been shown to improve ovarian structure and function through paracrine mechanisms that reduce apoptosis, modulate inflammation, stimulate angiogenesis, and promote follicular survival. Among these cell sources, Wharton's jelly-derived MSCs (WJ-MSCs) have demonstrated particularly promising regenerative properties. In a chemotherapy-induced mouse model of premature ovarian failure, Deng et al. reported that human umbilical cord MSC transplantation restored ovarian function by improving estradiol and FSH levels, increasing ovarian weight, enhancing follicular development, and recovering fertility, effects that were largely attributed to paracrine signaling (44). Encouraging results have also been reported in

clinical studies. Igboeli et al. observed increased ovarian volume, improved estradiol production, restoration of menstruation, and relief of menopausal symptoms after intraovarian transplantation of autologous bone marrow-derived MSCs in women with POF (45). Similarly, Mashayekhi et al. demonstrated menstrual recovery together with modest improvements in ovarian reserve markers following adipose-derived MSC therapy (46). Despite variability in hormonal responses among studies, restoration of menstrual function has consistently emerged as the most reproducible indicator of partial ovarian recovery, in agreement with the findings of the present study.

Evidence regarding the combined use of in vitro activation (IVA) and MSC therapy remains limited but is encouraging. Li et al. demonstrated that drug-free IVA combined with three-dimensional bioprinted adipose-derived stem cells significantly improved follicular development, angiogenesis, granulosa cell proliferation, and ovarian endocrine function in a rat model of primary ovarian insufficiency through activation of the PI3K/AKT signaling pathway (47). These findings support the hypothesis that combining follicular activation with regenerative cell therapy may produce synergistic effects by simultaneously stimulating dormant follicles and improving the ovarian microenvironment.

Taken together, our findings provide preliminary clinical evidence that combining WJ-MSC transplantation with IVA is a feasible and well-tolerated regenerative strategy for women with POF/POI. Although the favorable safety profile and sustained restoration of menstrual function are encouraging, larger randomized studies with longer follow-up are required to confirm efficacy, optimize treatment protocols, and determine the optimal cell source, dose, and adjunctive therapies for enhancing ovarian regeneration.

Conclusion

This phase I clinical trial provides preliminary evidence suggesting that the combined application of Wharton's jelly-derived mesenchymal stem cell transplantation and in vitro ovarian activation is a safe and potentially beneficial therapeutic approach for patients with premature ovarian failure. The absence of treatment-related adverse events throughout the study period supports the safety and tolerability of this intervention. Notably, a considerable proportion of participants experienced resumption of regular menstrual cycles, alleviation of menopausal symptoms, and improvements in endocrine parameters as well as endometrial thickness, indicating a possible functional response to therapy.

Although changes in AMH and estradiol levels did not reach statistical significance, the observed directional trends may reflect subtle biological activity and suggest a potential therapeutic effect that warrants further investigation. Overall, these findings support the hypothesis that combining regenerative cell therapy with ovarian activation strategies may enhance ovarian function and offer a novel therapeutic avenue for women with POF/POI.

Future optimization of this treatment protocol is necessary to improve clinical outcomes. This includes refinement of patient selection criteria, adjustment of WJ-MSC dosing and administration timing, and standardization of the IVA procedure. Furthermore, larger multicenter randomized controlled trials with

longer follow-up periods are essential to confirm efficacy, evaluate reproductive outcomes, and establish long-term safety.

In conclusion, this study supports the emerging role of regenerative medicine in reproductive endocrinology and highlights the potential of combined WJ-MSC and IVA therapy as a promising strategy for addressing the complex and currently limited treatment options for POF/POI.

Abbreviations

AMH Anti-Müllerian hormone

E2 Estradiol

FSH Follicle-stimulating hormone

LH Luteinizing hormone

POF Premature ovarian failure

WJ-MSC Wharton's Jelly-derived Mesenchymal Stem Cell

Declarations

Funding: This study was supported by a grant from Mehr Infertility Institute, Rasht, Iran.

Conflict of interest: All authors state that there is no conflict of interest.

Ethics approval and consent to participate: This project entitled “Unlocking Dormant Ovarian Function: A Phase I Clinical Trial of Regenerative Stem Cell Therapy Combined with In Vitro Ovarian Activation” has been approved by ethical committee at Guilan University of Medical Sciences in 2021/07/05 with this approval number: IR.GUMS.REC.1400.325 (IRCT NO. IRCT20211021052828N1). Written informed consent was obtained from all participants.

Consent to participate: Written informed consent was obtained from all participants prior to enrollment in the clinical trial

Consent for publication: Not applicable

Availability of data and material: All data are available by corresponding author

Code availability: Not applicable

Authors' contributions:

MM: Study design, surgery and intervention, recruiting and follow up the patients

EV: Corresponding, conceptualization, study design, data analysis, writing the initial draft, verification of the final version of manuscript, submitting the manuscript

AR: Recruiting and follow up the patients

ZB: Conceptualization, verification of the final version of manuscript

ZZB: Conceptualization, writing the initial draft, verification of the final version of manuscript

SM: Data analysis, verification of the final version of manuscript

MV: Conceptualization, study design, verification of the final version of manuscript

NA: Conceptualization, verification of the final version of manuscript

TZY: surgery and intervention, recruiting and follow up the patients

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Figures

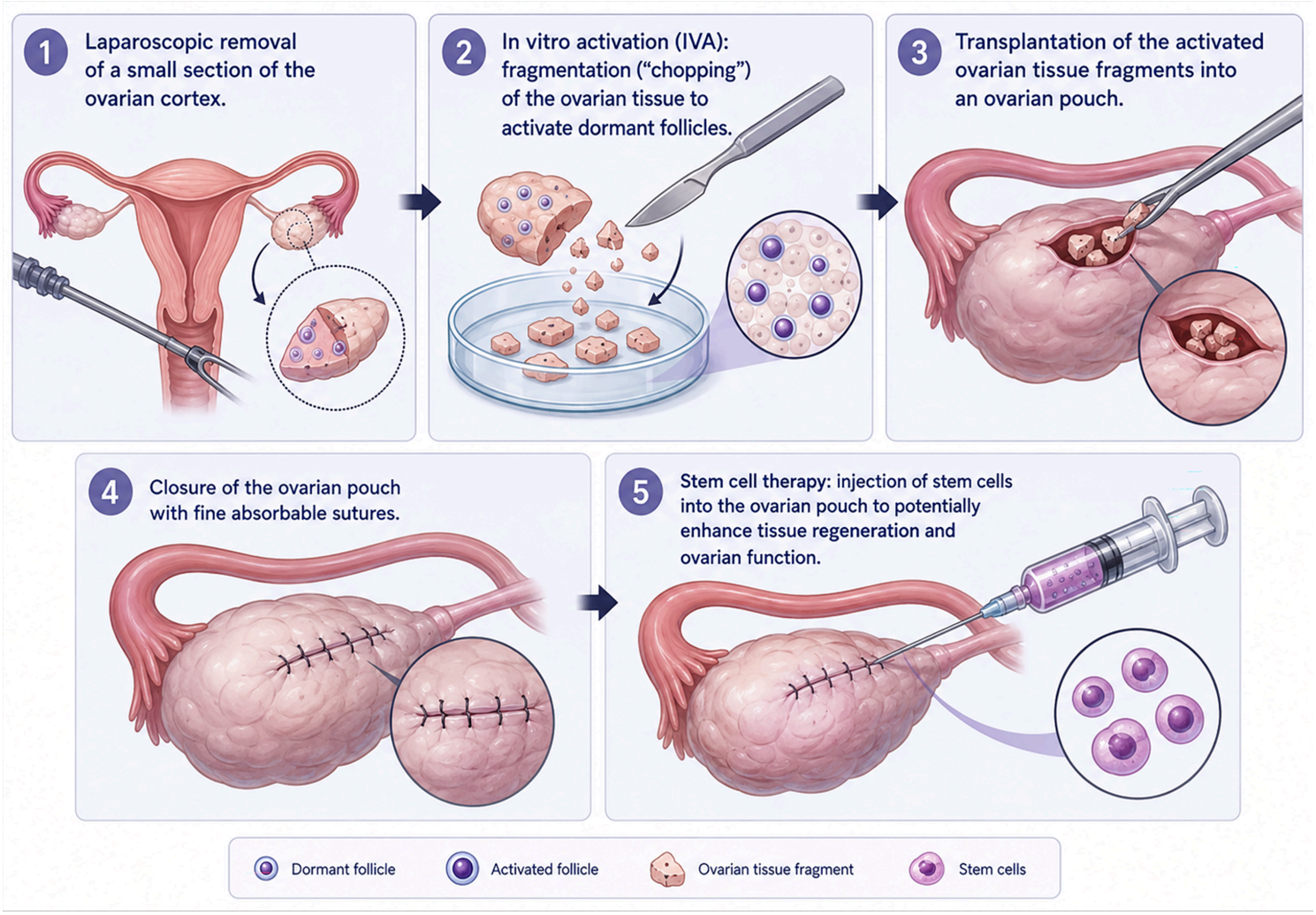


Figure 1

Graphical abstract

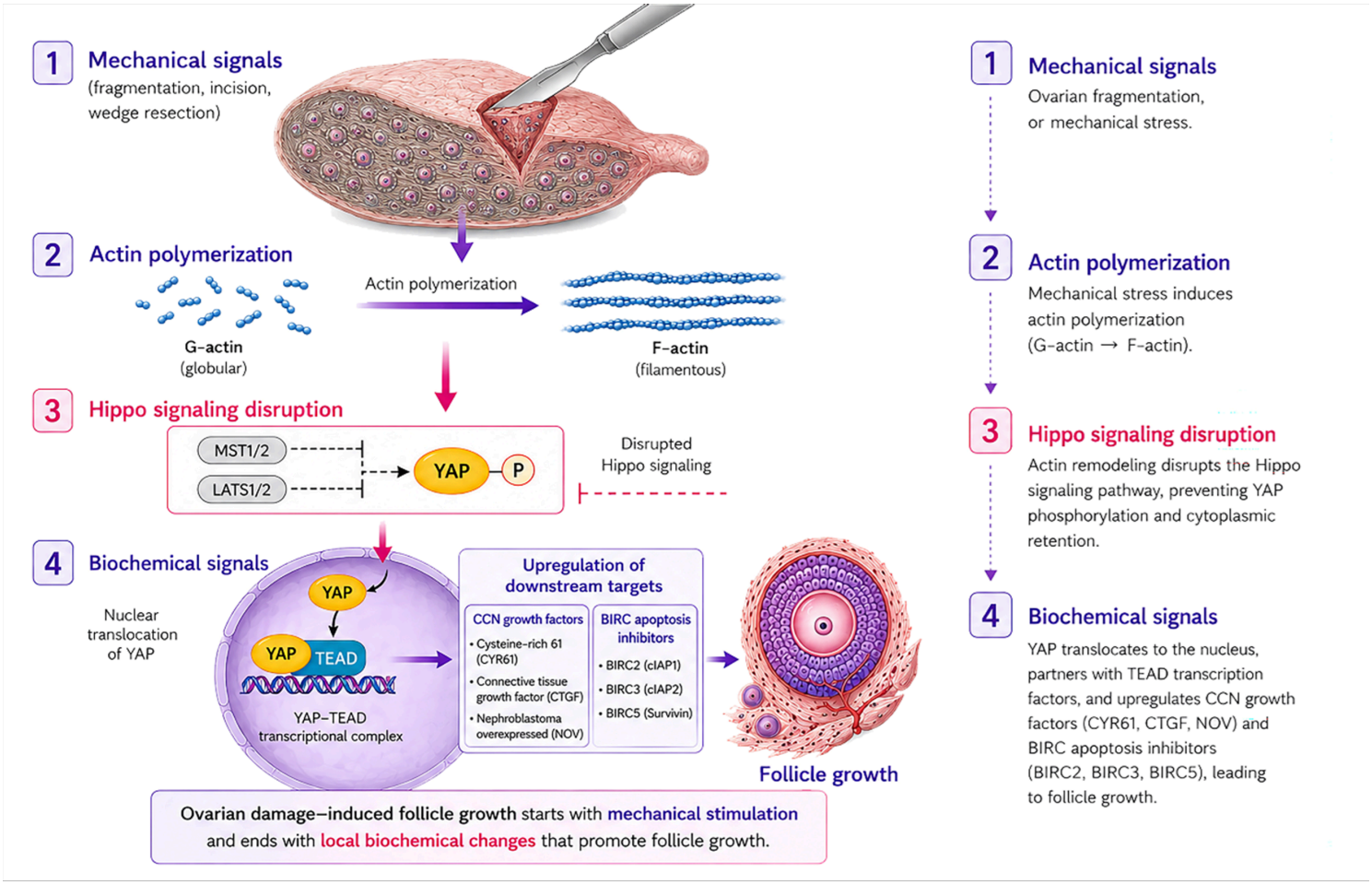


Figure 2

The rationale for applying laparoscopic ovarian chopping was based on the concept that [1] mechanical stress can promote follicular development by modulating Hippo signaling. Mechanical disruption of the ovarian cortex has been reported to reorganize [2] the actin cytoskeleton, [3] reduce Hippo pathway activity, and facilitate the nuclear accumulation of YAP. Activated YAP then interacts with TEAD transcription factors, [4] leading to increased expression of growth-promoting and anti-apoptotic genes, including members of the CCN and BIRC families. These downstream molecular events are thought to enhance follicle survival and growth, providing a biological basis for the mechanical intervention evaluated in the present study.

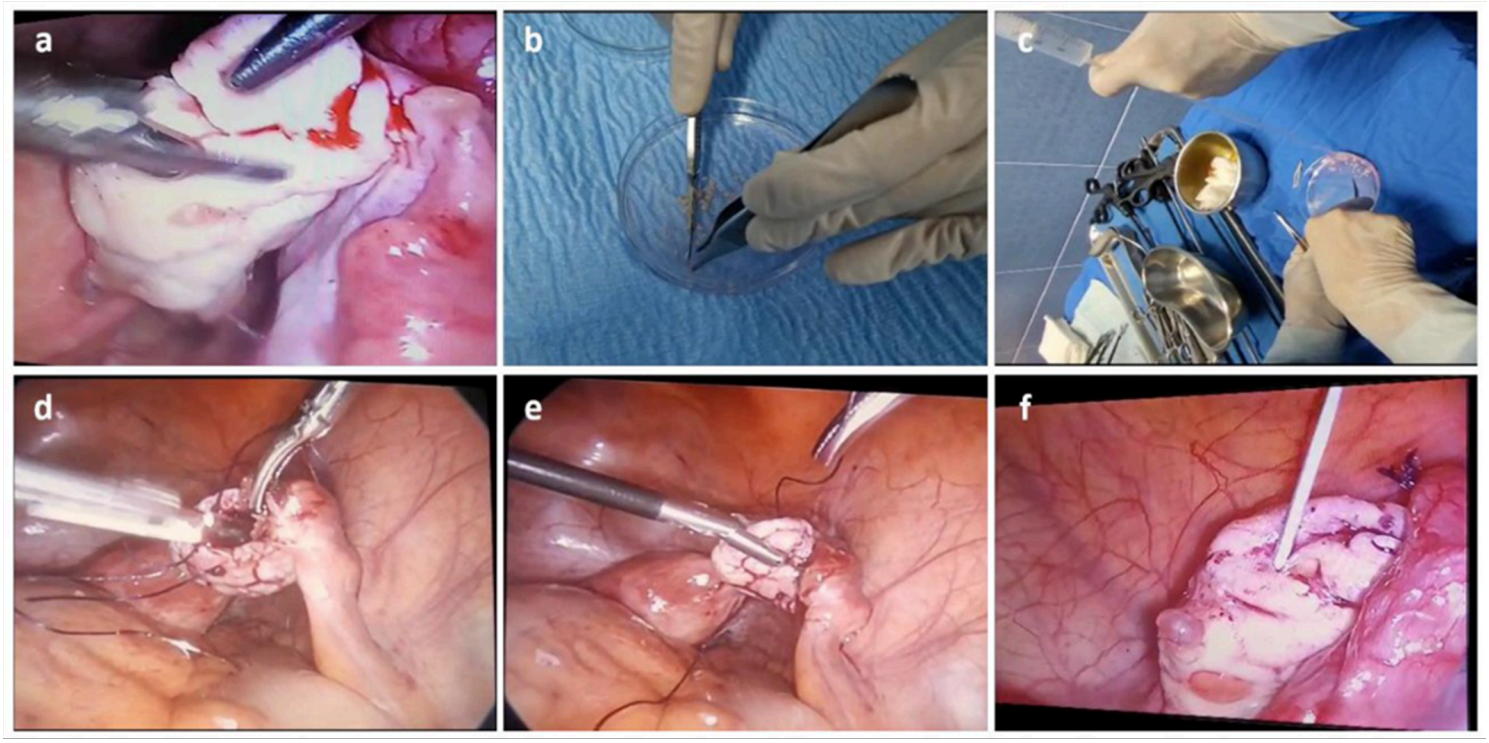


Figure 3

Surgical procedure. a: removal of a small section of the ovary; **b**: mincing the section into small pieces; **c**: transferring of the fragmented tissue using a cannula; **d**: transplantation of the fragmented tissue into the ovary pouch; **e**: stitching the ovary together; and **f**: injection of the stem cells into the ovary.

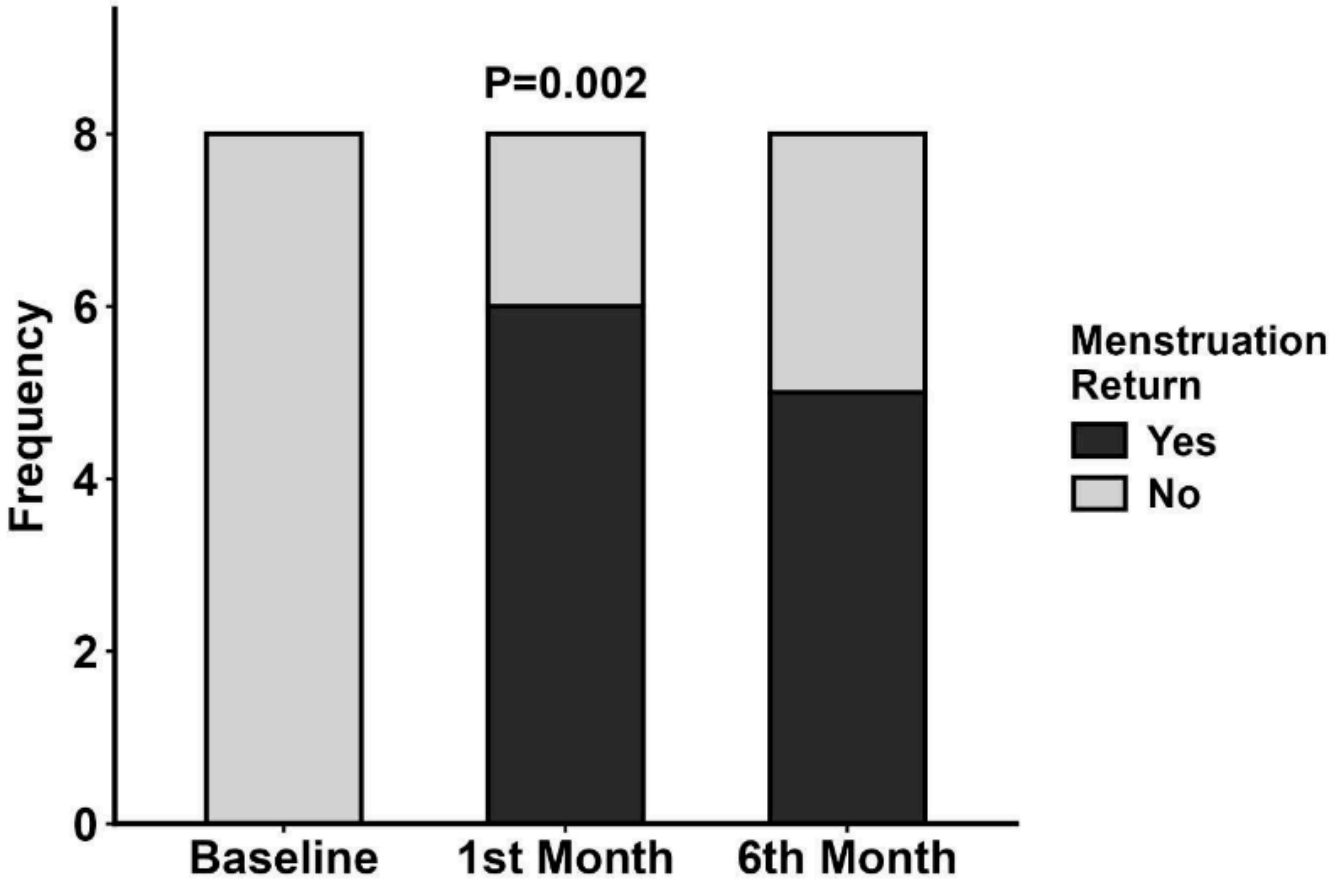


Figure 4

Menstruation return. Comparison of the menstruation return in women with POF at baseline, and 1 and 6 months after the treatment with stem cells along with IVA technique. P value was calculated by Cochran's Q test.

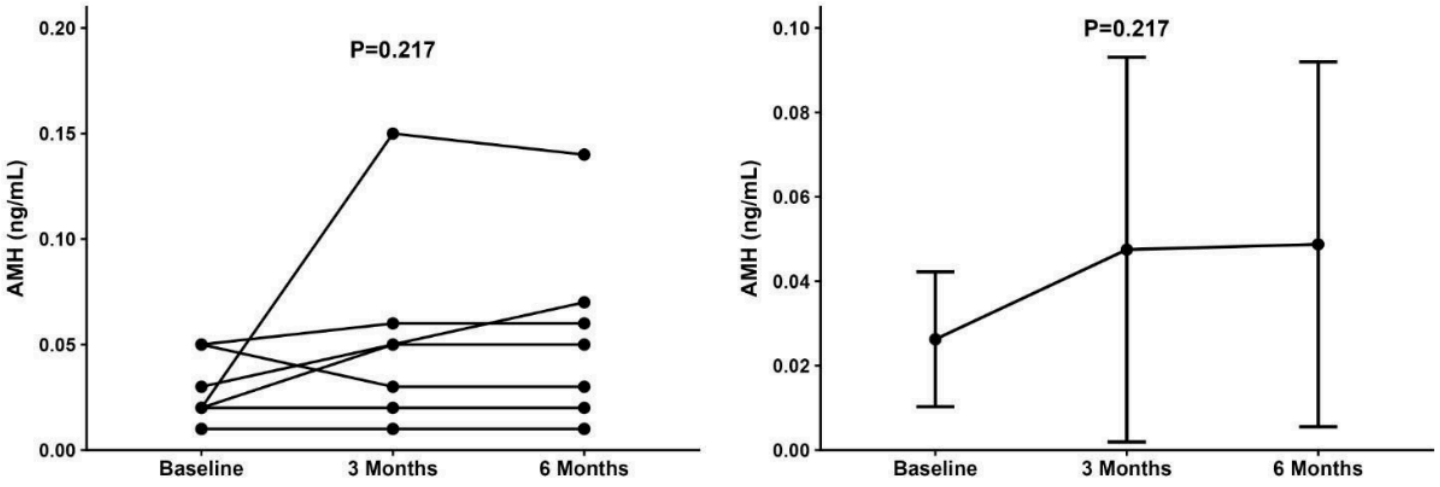


Figure 5

The serum level of AMH. The changes in AMH at baseline, and 3 and 6 months after the treatment. The injection of WJ-MSC along with IVA technique had no statistically significant effect on the serum level of AMH in patients with POF (P= 0.217).

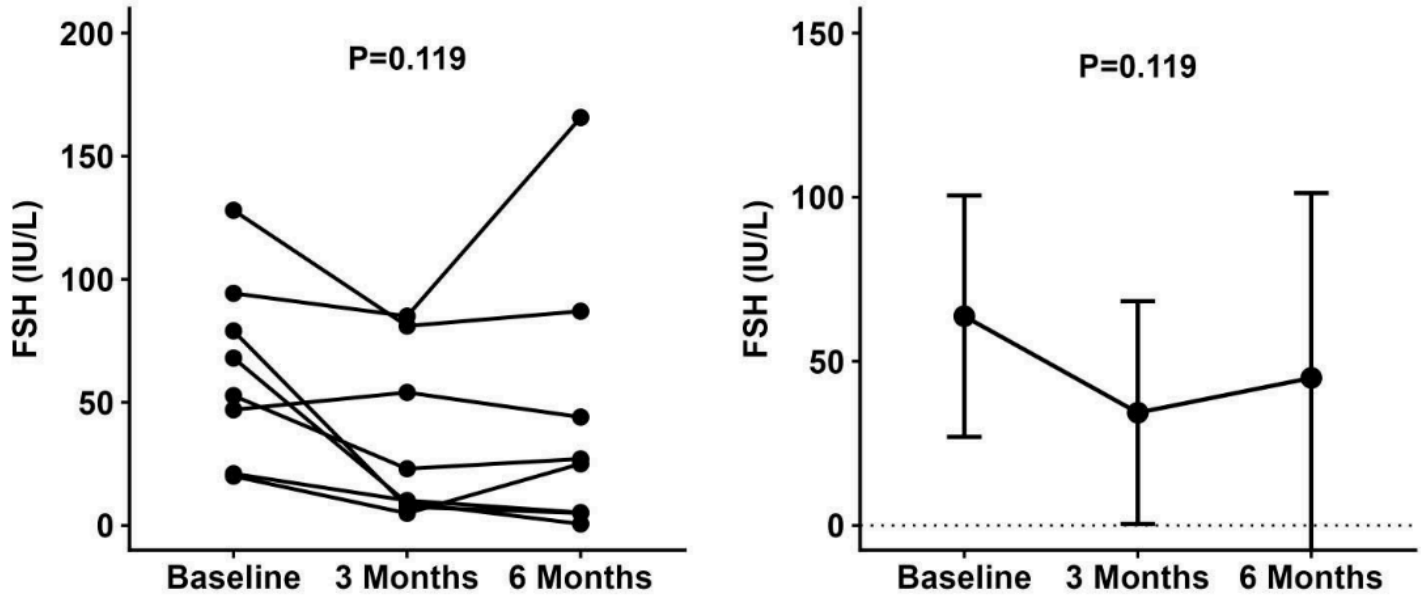


Figure 6

The serum level of FSH. The changes in the serum level of FSH at baseline, and 3 and 6 months after the treatment. The injection of WJ-MSC along with IVA technique had no statistically significant effect on the serum level of FSH in patients with POF (P= 0.119).

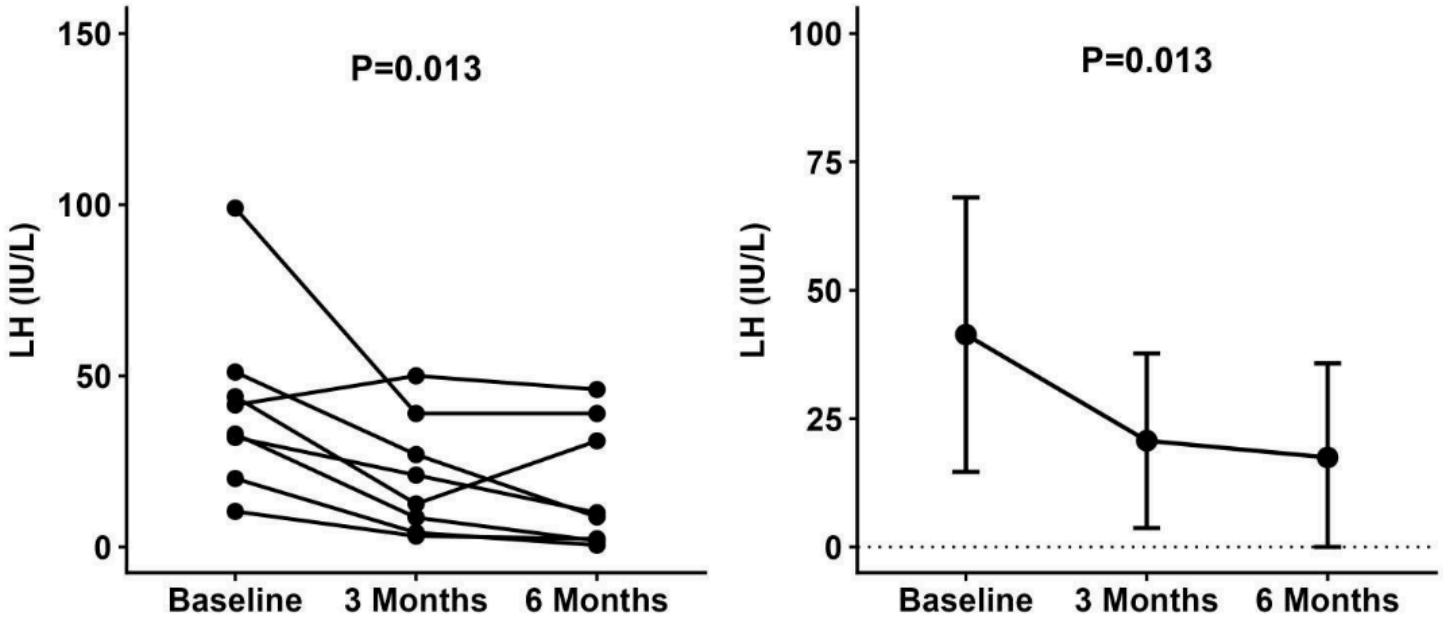


Figure 7

The serum level of LH. The changes in serum level of LH at baseline, and 3 and 6 months after the treatment. The injection of WJ-MSC along with IVA technique had statistically significant effect on serum LH level in patients with POF (P= 0.013).

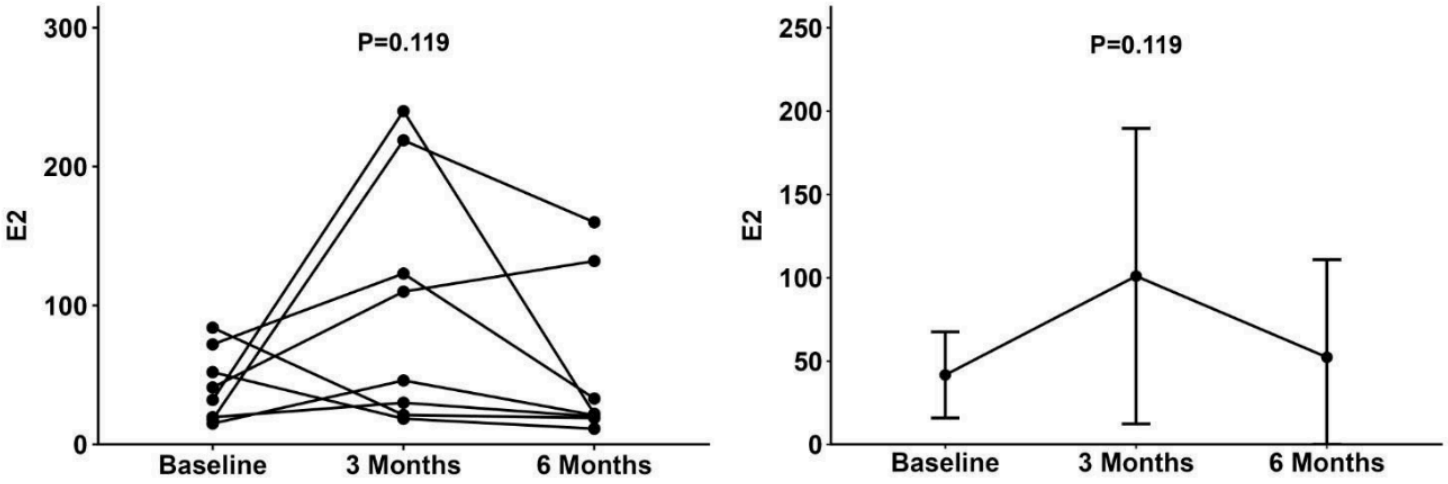


Figure 8

The serum level of E2. The changes in serum level of E2 at baseline, and 3 and 6 months after the treatment. The injection of WJ-MSC along with IVA technique had no statistically significant effect on the serum LH level in patients with POF (P= 0.119).

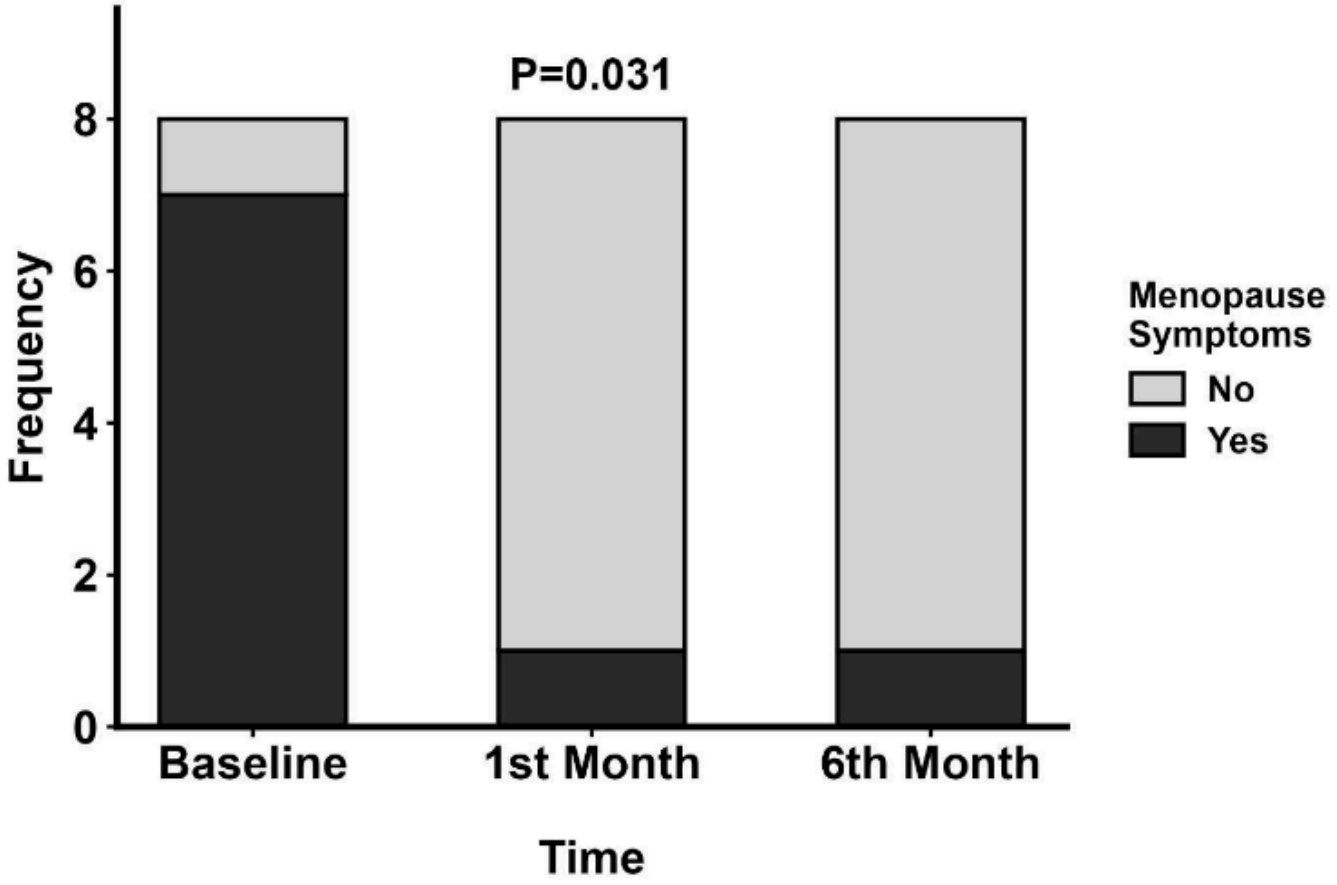


Figure 9

Menopause symptoms. Comparison of menopause symptoms in women with POF at baseline, and 1 month and 6 months after the treatment with stem cells and IVA technique. P value is calculated by McNemar's test.

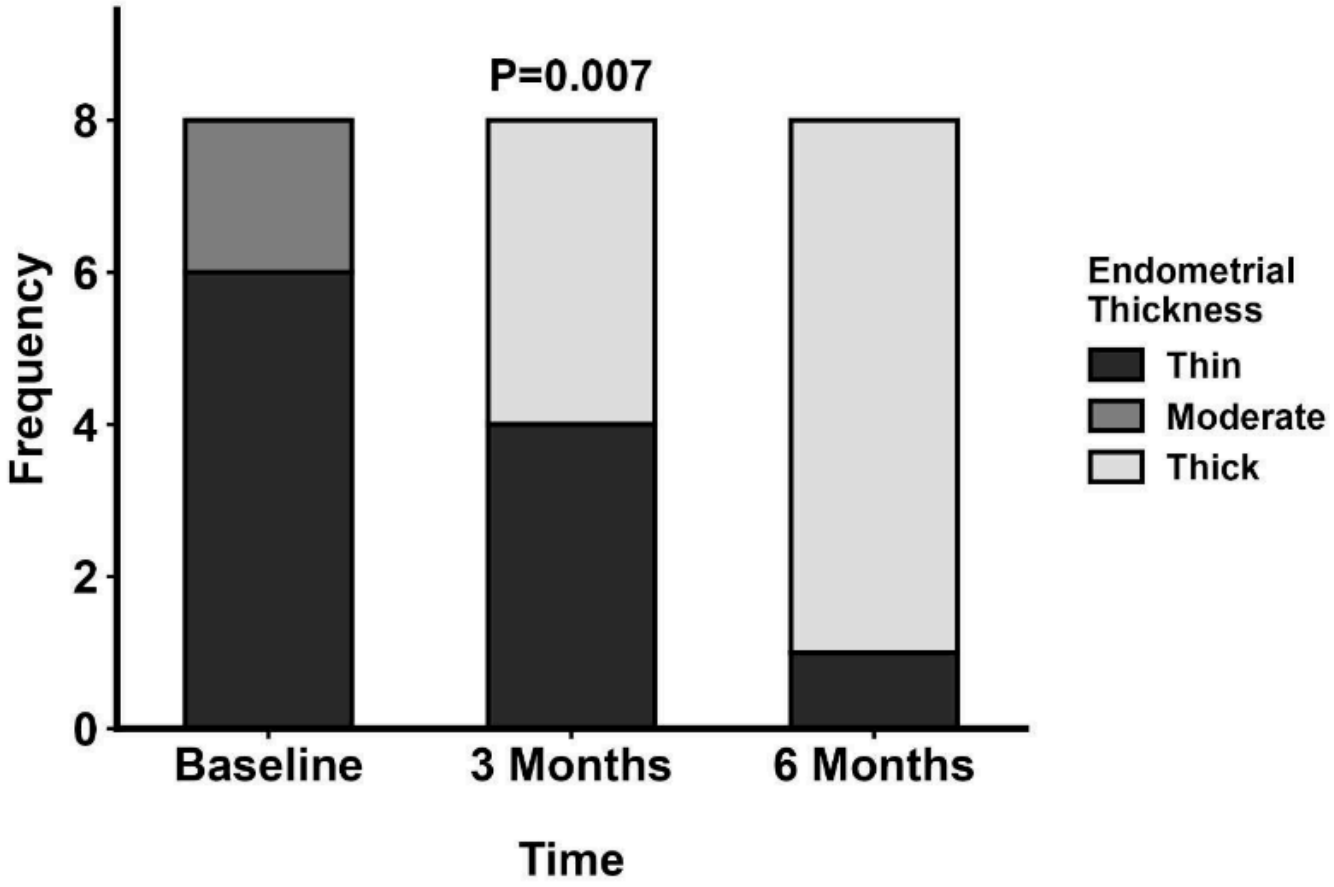


Figure 10

Endometrial thickness. Comparison of endometrial thickness in women with POF at baseline, and 3 and 6 months after the treatment with stem cells along with IVA technique. P value is calculated by chi-square test. Endometrial thickness: thin (<7 mm), moderate (8-13 mm), and thick (>14 mm).