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ORIGINAL ARTICLE

Intraovarian Administration of Autologous Menstrual Blood Derived-Mesenchymal Stromal Cells in Women with Premature Ovarian Failure

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Background. Premature ovarian failure (POF) is a well-known cause of infertility, particularly in women under the age of 40. POF is also associated with elevated gonadotropin levels, amenorrhea and sex-hormone deficiency.

Aim of the study. In this study, the therapeutic potential of autologous mesenchymal stromal cells obtained from menstrual blood (Men-MSCs) for patients with POF was evaluated.

Methods. 15 POF patients were included in the study. The cultured Men-MSCs were confirmed by flow cytometry, karyotype, endotoxin and mycoplasma and were then injected into the patients' right ovary by vaginal ultrasound guidance and under general anesthesia and aseptic conditions. Changes in patients' anti-Müllerian hormone (AMH), antral follicle count (AFC), follicle-stimulating hormone (FSH), luteal hormone (LH), and estradiol (E2) levels, as well as general flushing and vaginal dryness were followed up to one year after treatment.

Results. All patients were satisfied with a decrease in general flushing and vaginal dryness. 4 patients (2.9%) showed a spontaneous return of menstruation without additional pharmacological treatment. There was a significant difference in AFC (0 vs. 1 ± 0.92 count, p value ≤0.001%), FSH (74 ± 22.9 vs. 54.8 ± 17.5 mIU/mL, p-value ≤0.05%), E2 (10.2 ± 6 vs. 21.8 ± 11.5 pg/mL p-value ≤0.01%), LH (74 ± 22.9 vs. 54.8 ± 17.5 IU/L, p-value ≤0.01%) during 3 months post-injection. However, there were no significant changes in AMH (p-value ≥0.05%). There were also no significant differences in assessed parameters between 3 and 6 months after cell injection.

Conclusion. According to the findings of this study, administration of Men-MSCs improved ovarian function and menstrual restoration in some POF patients.

Key Words: Cell therapy, Clinical trial, Infertility, Premature Ovarian Failure, Premature Menopause, Menstrual blood stromal cells.

Introduction

Premature ovarian failure (POF) is a complex condition that affects 1–2% of women at some point in their reproductive lives (1). This endocrine problem impacts a woman's physical and psychological health (2,3). Women often experience this diagnosis as a sudden, life-changing shock, leaving them feeling out of sync. POF has been linked to environmental, genetic, metabolic, autoimmune

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and iatrogenic factors (4–6). Long-term hormone replacement therapy (HRT) with Gonadotropin-releasing hormone (GnRH)-agonists, estrogens, luteal hormone(LH)-releasing hormone analogues, corticosteroids, and human menopausal gonadotropin (HMG) has been the standard of care for POF patients in the past (7,8). Hormone therapy is typically used to address menopausal symptoms including sweating and vasomotor flushing, and is unlikely to be effective in curing these patients' infertility, (9,10). Infertility caused by POF requires more than hormone therapy, and long-term HRT has been linked to an increased risk of stroke, breast cancer, and heart attacks.

The most effective interventions in treating POF-related infertility are attempts to induce and raise these individuals' ovulation rates. However, the treatments used thus far have not been effective, and *in vitro* fertilization using donor oocytes has had the most success in these patients (11–15). Numerous studies have documented the advantages of using stem cells to treat infertility (16–20). Mesenchymal stem cells (MSCs) promote cellular recruitment, angiogenesis, regeneration, remodelling and immune cell activation or repression (21). MSCs can be harvested from different tissues and sources (16,17,22). Numerous research studies with animal models have demonstrated the usefulness of stem cells in POF (23–27). The findings show that stem cells have been successful in increasing ovarian functional reserve and the number of follicles. Additionally, MSCs were found to reduce FSH levels while increasing estrogen and vascular endothelial growth factor levels (28,29).

In 2007, a unique stem cell population, known as Men-MSCs, was introduced to regenerative medicine (30). Men-MSCs have higher rates of proliferation, self-renewal, and capacity for multiple differentiations compared to MSCs from other sources (31–34). This source is now more widely used and accepted because of the additional benefits it affords in terms of a quicker, safer and less invasive method of collection and no ethical issues (34–36). Men-MSCs and their derivatives have been used in various preclinical and clinical studies for illnesses like diabetes mellitus (37,38), multiple sclerosis (31), Asherman's syndrome (18), liver failure (39–41), myocardial infarction (42,43), Alzheimer's disease (44) and COVID-19 (45). In animal studies, transplantation of these cells has shown improvement in ovarian dysfunction and endometrial diseases (46–49). These cells effectively reduced granulosa cell apoptosis and ovarian interstitial fibrosis, resulting in increased follicular numbers and normalization of sex hormone levels (48,50). A recent clinical study conducted by this group showed a significant improvement in the ovarian function of those with poor ovarian response (20). Based on previous research and potential features of Men-MSCs, this study aims to assess the therapeutic potential of these cells in women with POF.

Materials and Methods

Study Design and Participants

This study was registered with the Iranian Registry of Clinical Trials (IRCT20180619040147N1). It was also approved by the Biomedical Research Ethics Committee of the Academic Center for Education, Culture, and Research (ACECR, Tehran, Iran). Every patient involved in the study was consulted and signed informed consent forms before participating. The current study was conducted at Avicenna Fertility Center and Avicenna Research Institute (Tehran, Iran). All the procedures in this clinical trial were performed according to the Declaration of Helsinki, Good Clinical Practice and Good Manufacturing Practice (GMP) guidelines.

15 POF women between the ages of 25 and 40 who had experienced amenorrhea for at least a year participated in the study. Their FSH level was $\geq$40 IU/L on two separate ocassions within four weeks, and their AMH level was less than 1 ng/mL. All of the patients had normal liver function tests (SGOT and SGPT), thyroid hormone levels (TSH and FT4), prolactin, BUN, creatinine, and fasting blood sugar levels. Their infectious disease tests (HIV, HCV, HBS Ag, and VDRL) were all negative. Furthermore, their coagulation factors (PT, PTT, BT, CT) and blood biochemistry levels of sodium, potassium, calcium, and phosphorus were all within normal limits (Table 1). It was assured that the patients had not used any hormonal medicine for at least 4 months prior to the study's start date. All individuals were evaluated for genital abnormalities such as endometrioma, ovarian cysts, or other reproductive system disorders prior to the trial. Women with uterine disorders or male factor abnormalities (sperm count <5 million/mL, normal morphology <2%) were excluded from the study. There was no history of POF on the patient's mother's side of the family. All patients complained of vaginal dryness and overall flushing as a result of hormonal imbalance. The BMI, FSH, and AMH levels of the 15 women who met the inclusion criteria were comparable, and they were approximately the same age. Conjugated Estrogen 1.25 mg tablets (Ferrer International Co., Spain) were administered to all patients for 25 days. From day 16–25, Duphaston 10mg tab (Abbot, Switzerland) was prescribed to collect menstrual bleeding samples.

Preparation and Culture of Men-MSCs

Menstruation happened prior to the administration of medicine. On the second day of menstruation, the patient's menstrual blood was collected using a sterile DivaCup (Diva International Co., Lunette, Finland) and the number of antral follicles was measured by vaginal ultrasonography (Honda 2000-5 MHz, Japan). The samples were then transferred to a collecting tube containing GMP-grade Dulbecco's Modified Eagle's Medium-F12

Table 1. Patient's Inclusion and Exclusion Criteria

Inclusion criteria	Exclusion criteria
20–39 years old	Congenital abnormalities of the reproductive tract
Amenorrhea for at least 1 year	Autoimmune disorders
FSH ≥40 IU/L	Asherman's syndrome
AMH <1 ng/mL	Family history of ovarian tumors
Normal Karyotype	History of cancer, chemotherapy, radiotherapy
Normal HSG (Hysterosalpingography)	Known history of HIV, Hepatitis B, C, Syphilis
Normal Endocrine Tests (TSH, FT4, PRL, FBS)	Endometriosis
Normal Coagulation Factors (PT, PTT, BT, CT)	Sperm count <5 million/ml, Normal morphology <2 %
Normal Serum Biochemistry Tests (Na, K, Ca, P)	

(DMEM-F12, Gibco, UK), 2.5 μg/mL Fungizone, 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.5 mmol EDTA in phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} (GIBCO, UK). The specimens were immediately transported to the class-B clean room for MSC isolation and culture. The isolation, culture, and release procedures were carried out as described in our previous paper (20).

Safety and Quality Control of Men-MSCs

Men-MSCs were produced in the manner previously described (20). The quality of the Men-MSCs was tested for fungal and bacterial contamination using sterility test by direct inoculation, mycoplasma assay by both culture and polymerase chain reaction (PCR) (in accordance with USP chapter [63] Mycoplasma Tests), endotoxin test (in accordance with USP chapter [85] Bacterial Endotoxins Test), Karyotyping using routine G-banded chromosome analysis, and immunophenotyping evaluation using CD73, CD105, CD90, and CD45 expression analysis. All the cell quality-control tests were preceded using guidelines of the International Conference of Harmonization *Q*2 (ICH *Q*2).

Intraovarian Injection of Stem Cells

On the injection day, the cultured cells were trypsinized, recounted, and suspended in normal saline containing 10% human serum albumin. The final product had a density of 20×10^6 cells/mL. 300 μL of the prepared suspension was injected intra-vaginally with the help of vaginal ultrasonography (Honda 2000-5 MHz, Japan) into the right ovary of the patients under general anaesthesia with midazolam (Caspian pharmaceutical Co. Iran) and fentanyl (Caspian pharmaceutical Co. Iran). On the injection day, AMH level, AFC, and ovarian function were checked twice.

Evaluation and Clinical Assessments of Patients

The patients were closely monitored to evaluate the safety and efficacy of treatment. For up to one week, all patients were monitored for symptoms such as fever, vomiting, high blood pressure, arrhythmia, or vaginal bleeding. To assess the safety, they were also checked for side effects such as ovarian abscesses and neoplasms throughout the study using routine physical examination and vaginal ultrasonography. The main objective of this study was to see how stem cell administration affected reoccurring menstruation, changes in hormone levels (FSH, AMH, LH, and E2), and AFC three months after stem cell administration. During these three months, the patients were permitted to engage in sexual activity. In addition to the recurrence of menstruation, they were tested for pregnancy. Changes in physical and emotional status, flushness, and vaginal dryness were also investigated.

Statistical Analysis

The quantitative results were analyzed using SPSS, version 21.0 software (IBM Corp., Armonk, NY). Non-parametric Tests (Wilcoxon signed Ranks) were used to compare the differences before and after injection. Data are presented as the mean (IQR). A p-value <0.05 was considered statistically significant. The analysis was performed by a blind statistics analyzer.

Results

Quality Control of Cultured Men-MSCs

The cultured cells showed a spindle-shaped fibroblastic morphology. The results obtained from immunophenotyping analysis of the cultured cells were positive for CD90 (98.5 ± 1.9%), CD73 (99.5 ± 0.5%), and CD105 (92 ± 2.35%) markers, and negative for hematopoietic marker CD45 (2 ± 1.02%). In addition, a normal karyotype pattern was obtained, and no evidence of microbial growth was present. No colony-forming unit (CFU) was observed in Gram stains either. Endotoxin samples showed no LAL clot formation. Also, samples analyzed by DNA amplification were negative for mycoplasma expression (Table 2).

Safety Evaluation Post-administration of Stem Cells in Patients

Patients were closely monitored for 4 hours after cells administration for side effects such as bleeding, excruciating

Table 2. Characterization of the Autologous Menstrual-Derived Stromal Cells

Parameter	Result
Total no. of injected cells	3×10^6
Cell Viability %	100
FACS ($CD90^+$) %	98.5 ± 1.9%
FACS ($CD105^+$) %	92 ± 2.35%
FACS ($CD73^+$) %	99.5 ± 0.5%
FACS ($CD45^-$) %	<10%
Mycoplasma Test	Not Detected
Sterility Test	Sterile
Bacterial Endotoxin	≤0.125 EU/mL
Karyotype by G-banding	Normal

pain, nausea, and discomfort. They were all discharged without complications. For two weeks, patients' daily phone checkups with a specialist revealed no clinical signs such as pain, fever, or infection. Throughout the study, no suspicious symptoms were discovered during the physical examination or intravaginal ultrasonography.

Effects of Men-MSCs on Mensuration Resumption, Hormone Levels, AFC, and Physical Condition of the Patients

This study enrolled 15 patients, 12 of whom completed it, and the first examination was 3 months after stem cell administration (Figure 1).

The clinical characteristics of the 15 POF patients are listed in Table 3. All of these women suffered from secondary amenorrhea. The average age, BMI, and duration of amenorrhea for the patients were 32.2 years old, 22.86 kg/m^2 and 2 years, respectively. All the patients were given 3×10^6 autologous Men-MSCs via right ovary injection. Participants reported no side effects during the first 24 hours or the first week after injection. Physical examination and vaginal ultrasonography at weeks 2, 3, 4, and at 12 months revealed no secondary complications. Menstruation returned in four patients (26%), and the menstrual cycles lasted an average of two cycles in these patients. FSH and LH secretion decreased significantly after injection (p value ≤0.05%). Even though E2 and AFC levels increased significantly (p-value ≤0.01%, p-value ≤0.001%, respectively), the amount of this increase was insufficient for a proper and safe pregnancy. However, an increase in E2, which is essential for the ovaries' proper function and quality, returned E2 levels to normal. Although AMH secretion increased slightly, the difference was not statistically significant (p-value ≥0.05%).

Six months after the injection, the second evaluation was scheduled. Three patients declined to participate in the study on their own volition. As shown in Figure 2, FSH and LH levels remained low in comparison to pre-injection levels (p-value ≥0.05%). E2 levels remained significantly higher (p-value ≤0.05%), AFC levels did not differ significantly, and there was no significant difference in AMH levels 6 months after injection (p-value ≥0.05%). When the results from 3 and 6 months were compared, it was discovered that AMH secretion levels had decreased (p-value ≥0.05%). During these three months, there were no significant changes in FSH, LH, E2 levels, or AFC (Figure 2). The first visit was on the second or third day of bleeding in patients who had a spontaneous recurrence of menstruation. Under the observation of a specialist, these four patients began Induction Ovulation (I/O) treatment, including Letrozole 2.5 mg tab (Aburaihan, Iran) TDS for 5 days (beginning on the third day of the menstruation cycle), as well as 75 units of HMG (IBSA, Switzerland) injected on days 8 and 9 of their cycle, and it was increased or continued if necessary. The first patient was 31 years old, had one failed pregnancy and her last menstrual cycle was about 2 years ago. Menstruation resumed one month after stem cell administration and lasted for two cycles. One 18 mm follicle was detected on 18^{th} day of the cycle after starting I/O, but pregnancy did not occur. The second patient, who experienced a recurrence of menstruation, was also 31 years old. This patient had suffered from amenorrhea for 8 years; menstruation returned one month after the intervention and lasted 8 cycles; two antral follicles were obtained. Despite the fact that her follicles were large enough for pregnancy in her second cycle, she did not conceive. The third patient had a history of one successful pregnancy and had been suffering from amenorrhea for 13 years. Her menstrual cycles returned two months later, lasting two cycles, and her AFC was one in each cycle, increasing in size to 14 mm. Finally, the fourth patient was 35 years old and had been suffering from amenorrhea for the previous four years. Her menstrual cycles returned two months after the injection and lasted three months. Furthermore, three antral follicles were formed, but they were insufficiently large. Menstruation did not resume in the other 11 patients.

Discussion and Conclusions

For the first time, this study looked into the therapeutic effects of intra-ovarian injection of autologous Men-MSCs in POF patients. During this study, no abnormal systemic or local clinical signs or side effects were observed, implying that the procedure was safe. Despite the fact that this intervention did not result in a desirable pregnancy outcome, significant and promising changes in other assessed parameters opened a new window into the treatment of POF women's infertility. One of these modifications was a significant increase in AFC levels three months after cell administration. We presume these patients had a few pre-antral follicles in their ovaries and that Men-MSCs aided in their development and maturation. Other changes occurred in the levels of FSH and E2, which are critical for the function of the ovaries and indicators of POF diagno-

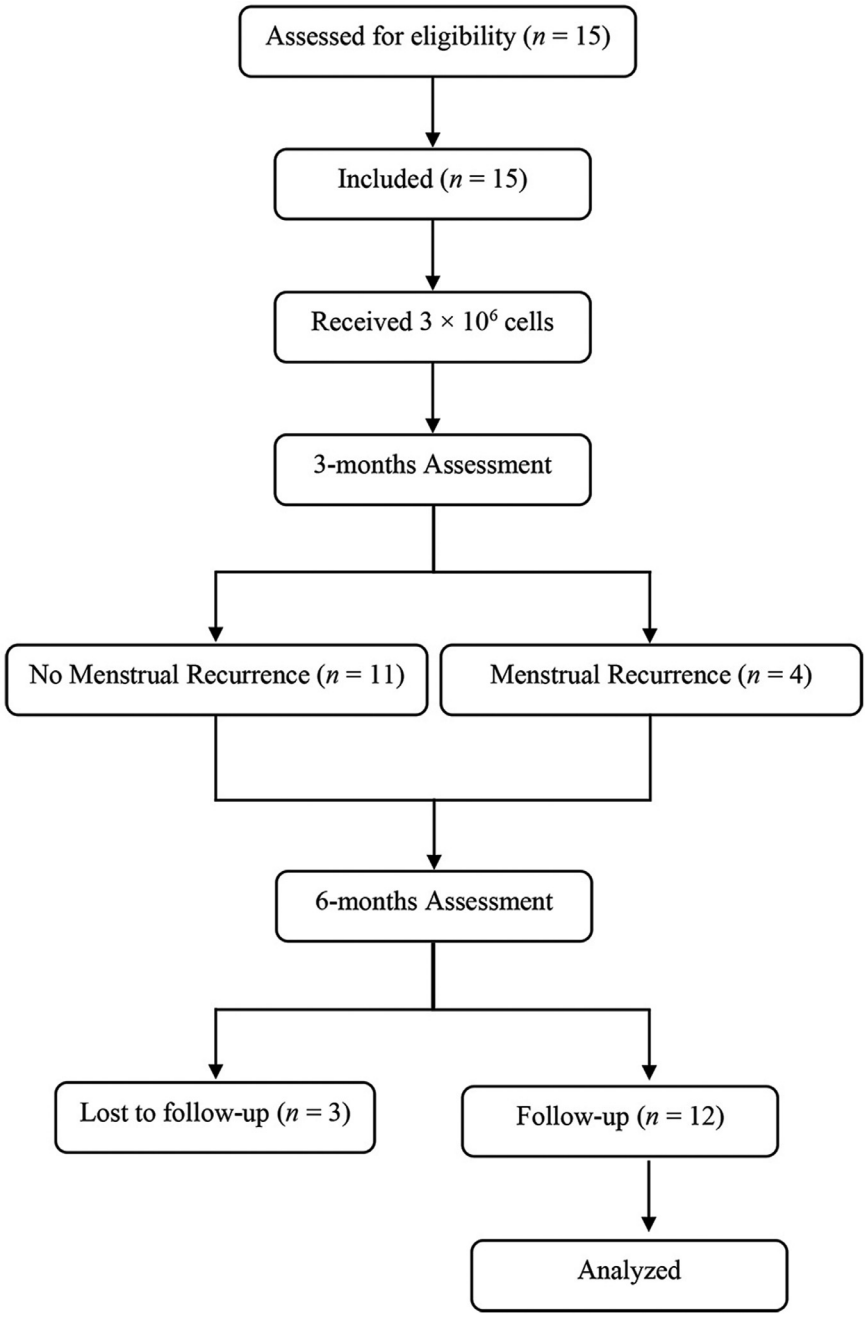

Figure 1. Flow Chart of POF Patient's Participants.

sis. In our study, FSH levels decreased while the levels of E2 increased. These cells are thought to differentiate into granulosa cells, and the process of regeneration, evolution, and secretion of inhibitory hormones keeps FSH levels low and prevent follicular evacuation, which explains why our FSH levels remained low and steady even after 6 months of treatment. During the study, there was also an improvement in the patients' mental and physical health. Vaginal dryness, excessive sweating, and constant flashness were recovered during our experiment, and we supposed that it was due to the gradual recovery of estrous cycles. Our patients' FSH levels also reduced and remained low even 6 months after cell administration. Although the differences in AMH were not statistically significant, an increase in hormonal levels indicated a promising approach to using these cells to improve the ovary function in POF patients.

To the best of our knowledge, our clinical trial is the only one that uses Men-MSCs to treat POF. Indeed, the efficiency of Men-MSCs had only been investigated using animal models so far. A research group detected these cells

Table 3. Clinical Characteristics and AFC and Hormonal Situation of POF Patients at Baseline

No.	Age (years)	BMI	Duration of amenorrhea (years)	LH (mIU/mL)	FSH (mIU/mL)	AMH (ng/mL)	E2 (pg/mL)	AFC
1	35	21.40	2	53	64	0.07	10	0
2	34	18.50	2	42	90	0.01	5	0
3	26	20.00	2	45	68	0.01	12	0
4	34	21.00	2	46	56	0.01	2	0
5	31	25.00	2	27	49	0.00	2	0
6	34	26.00	5	70	90	0.01	2	0
7	31	23.00	2	53	95	0.01	10	0
8	38	24.00	12	24	63	0.01	6	0
9	35	22.00	2	14	52	0.01	11	0
10	32	23.00	3	63	134	0.00	20	0
11	33	23.00	13	70	77	0.07	15	0
12	28	21.00	3	41	62	0.50	21	0
13	24	22.00	3	46	95	0.80	12	0
14	39	29.00	3	50	60	0.09	15	0
15	35	24.00	4	40	55	0.03	10	0

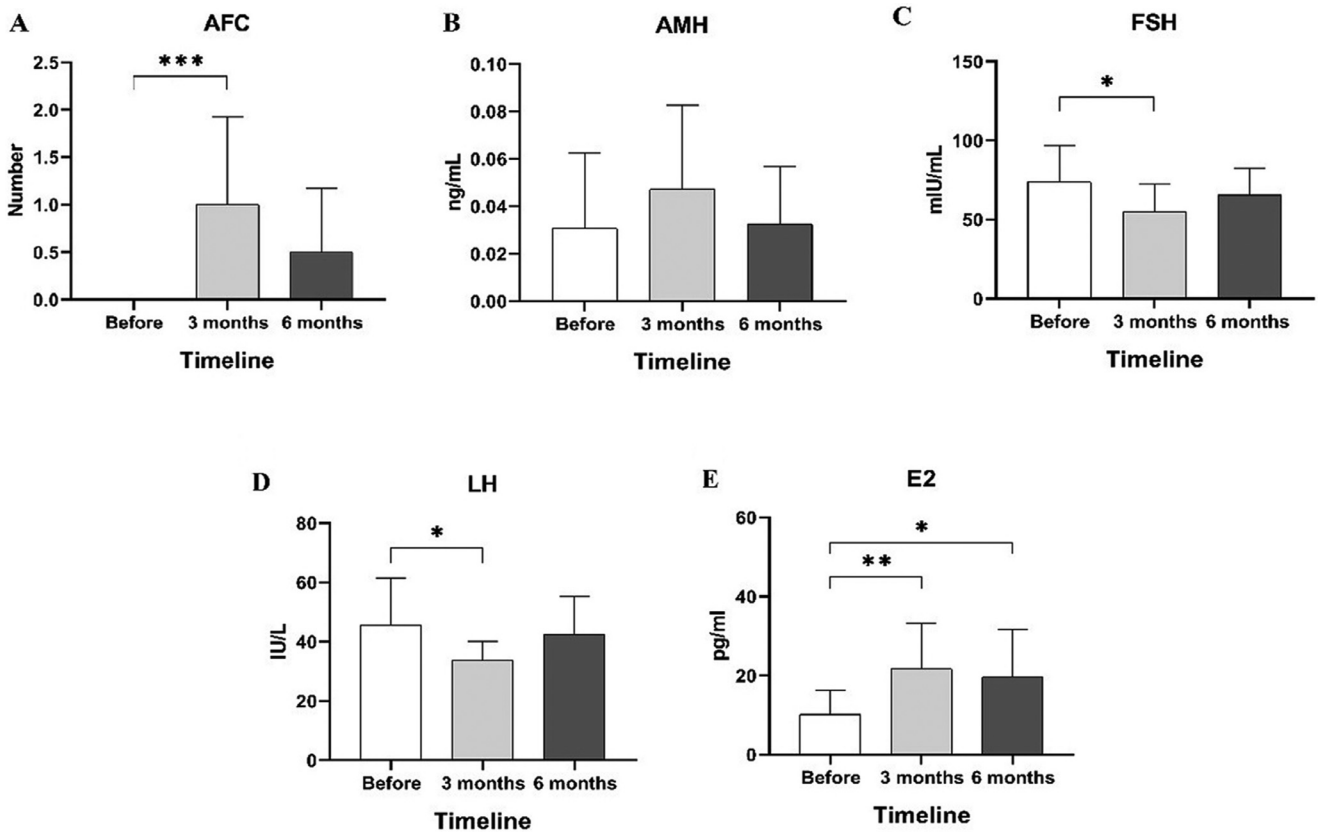

Figure 2. The Mean Value of AFC and Hormonal Changes at Base Line, 3 Months and 6 months Post Injection. A. The Antral Follicular Count showed a significant difference between pre-stem cell injection and 3 months post-injection (***p-value $\leq$0.001), but there was no significant difference between other times of evaluation, B. Although AMH levels showed a little increase 3 months after injection, there was no significant difference throughout the study C. FSH levels showed a significant difference between pre-injection and 3 months post-injection (*p-value $\leq$0.05%), but there was no significant difference between other timelines, D. LH levels decrease significantly after 3 months of injection (*p-value $\leq$0.05%) but there was no significant difference between other evaluation timelines, E. E2 increased significantly after 3 months (**p-value $\leq$ 0.01%), this increase stayed significant after 6 months (*p-value $\leq$ 0.05%), but no significant difference between 3 month and 6 months of the evaluation was seen.

in the ovaries using live imaging and immunofluorescent methods and demonstrated that this transplantation reduced the depletion of germline cells, indicating that these cells can restore the function of damaged ovaries (51). Transplanted stem cells are thought to be capable of residing in ovarian tissue and restoring ovarian function (52–54). This coverage of ovarian damage and function happens through the secretion of paracrine factors, salvaging the existing oocytes, and repairing ovarian niches (55,56); however, the mechanism underlying this remains unknown.

Noory P, et al. transplanted human Men-MSCs intravenously into a chemotherapy-induced POF model in rats. The histological findings revealed that the number of follicles had increased. Furthermore, in the treatment group, ovarian weight increased and approached that of the normal controls. One month after transplantation, the cells were discovered in the ovarian interstitium and granulosa cells. Their research concluded that these cells could be a viable and low-cost method for treating POF (47). Wang Z, et al. performed another study in which Men-MSCs were transplanted into POF models of mice through the tail vein. They also showed an increase in antral follicles and even pre-ovulatory follicles in the treatment group (48). Guo F, et al. conducted another study on the effects of Men-MSC in animal models of POF. The mice's general physical condition improved after Men-MSC transplantation, as did their body weight, coat, and appetite. They also found a decrease in FSH, an increase in AMH and E2 levels, and an increase in the number of secondary and antral follicles (19). Liu D, et al. obtained similar results in the same study (46). To support the effectiveness of Men-MSC in enhancing fertility, the development of a co-culture system with mouse preantral follicles and human Men-MSC has resulted in indices of folliculargrowth, increased survival rate, follicular diameter, antral formation, and the rate of *in vitro* maturation (57).

As mentioned above, cell-based therapies for POF have mainly focused on sources other than menstrual blood. Edessy and colleagues reported the first stem cell therapy for POF. They transplanted autologous bone marrow stem cells into ovaries by laparoscopy. They also experienced a 20% recovery of menstruation 3 months after the treatment, and one case conceived successfully (58). Gupta and colleagues reported the world's first baby born after intra-ovarian transplantation of autologous MSCs in a premenopausal 45 year-old patient. They reported an increase in AMH levels 2 months after treatment and discovered two follicles in each ovary. The patient became pregnant via embryo transfer and gave birth to a healthy baby (59). Recently, Mashayekhi and colleagues reported the first human clinical trial on autologous adipose tissue mesenchymal stem cell (AT-MSCs) transplantation by intra-ovarian injection as a treatment for idiopathic POF patients. They tested three doses (5, 10, and 15 $\times$ 10^6) in 9 patients. Their result showed menstrual resumption in 4 patients and reduced FSH levels one year after cell administration. The ovarian volume, AMH, and AFC showed no significant differences between cell groups (60). Igboeli and colleagues again used autologous MSCs to improve ovarian function in two FOP patients. Autologous stem cells were injected into the patients' right ovary, leaving the left ovary as a control. They reported approximately a 50% increase in the volume of the treated ovary compared to the contralateral ovary, with a 150% increase in serum estrogen levels compared to preoperative values. Significant improvement in their menopausal symptoms was also reported and both patients had menstrual episodes that lasted 1 year. No side effects were reported throughout the study (61). The differences in reported results could be attributed to the discrepancies in patient population, stem cell type and source, stem cell preparation method, dose and route of administration.

The mechanisms that determine the stem cells' effectiveness in regeneration and revitalizing of ovarian function, neo-oogenesis, and folliculogenesis are not entirely clear. Stem cells from different sources can have some effects on restoring ovarian function due to cellular and local signalling (62,63). Follicular renewal in ovaries is believed to be a result of ovarian stem cells in the tunica albuginea layer of the ovary, which can differentiate into granulosa and germ cells. Men-MSCs have been shown to have multilineage differentiation potential (46,64). Note that primordial follicles arise from granulosa cells (65,66). In addition, Men-MSCs are believed to improve the ovarian microenvironment by releasing VEGF, HGF, and IGF-1, growth factors, and cytokines which play a key role in tissue repair and regeneration (67,68). These factors are helpful for angiogenesis, anti-apoptosis, anti-inflammation, immunoregulation, and anti-fibrosis (22,36). It is also hypothesized that Men-MSCs can differentiate into multiple ovarian cells which can contribute to ovarian regeneration (69).

As previously described, using menstrual blood as a source for stem cells, has advantages over other stem cell sources with respect to aneasier, safer and non-invasive collection method. However, because they are a newly discovered source, mostly animal studies have been conducted using these cells for ovarian and uterine defects. Although it has not been studied in patients with POF, human studies using this source have demonstrated the safety and feasibility of Men-MSCs in infertility treatment of Poor Ovarian Responders and patients with Asherman syndrome (18,32). Given the improved state of the ovarian microenvironment in poor ovarian responders compared with POF patients, the role of appropriate dual interaction between the administrated stem cells and niche to allow for pregnancy is greatly enhanced. As a corollary, considering the ease of sampling and isolation of this source and based on the safety and efficacy of these cells, we conclude that these cells can provide and maintain a rich microenvironment for

the ovaries and follicles and have potential for therapeutic use in POF treatment. However, additional clinical studies with a more optimized procedure, such as higher doses or multiple doses, and then enrollment of a larger patient population in different stages of clinical studies can provide us with a more comprehensive understanding of the effects and benefits of Men-MSCs.

Conflicts of Interest

None of the authors received grants or outside funding in support of this study. The authors have no conflict of interests.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.arcmed.2022.12.015.

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