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Research Article

Bone Marrow Mesenchymal Stem Cell Therapy for Cyclophosphamide-Induced Premature Ovarian Failure in Rats

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Abstract

Premature ovarian failure (POF), often induced by cyclophosphamide (CP), leads to hormonal imbalance. Current treatments are ineffective at restoring fertility, creating a need for novel therapies. This study aimed to investigate the therapeutic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) in restoring ovarian function in a rat model of CP-induced POF. Thirty female rats were divided into control, POF (induced by CP injections), and treated group received a single intravenous injection of BM-MSCs (POF+BM-MSCs). After four weeks, hormonal levels, ovarian oxidative stress markers, and histopathological changes were analyzed. CP induction successfully created a POF model, evidenced by significantly decreased E2 ($P<0.001$); increased FSH, LH ($P<0.01$), elevated oxidative stress, thyroid dysfunction ($P<0.01$), and severe follicular degeneration. BM-MSC transplantation effectively reversed these effects, normalizing hormone profiles, reducing oxidative stress, restoring thyroid function, and improving ovarian follicular architecture. However, BM-MSC treatment did not significantly improve the CP-induced uterine damage. It was concluded that BM-MSC therapy demonstrates a strong protective effect against CP-induced POF. Despite its pronounced efficacy within the ovary, the therapeutic benefits were incompletely extended to the uterus under the employed treatment protocol. This suggests that further investigation into optimized delivery methodologies to ensure a comprehensive, whole-reproductive-tract reparative outcome.

Keywords: Bone marrow mesenchymal stem cells, Cyclophosphamide, Ovarian toxicity, Oxidative stress marker, Premature ovarian failure

Introduction

Premature ovarian failure (POF) is a diverse condition characterized by the termination of ovarian activity, along with increased levels of the follicle-stimulating hormone (FSH) and lower levels of estradiol (E2) [1,2]. Ovarian toxicity reduces follicle stores, causes menstrual irregularities, and leads to ovarian dysfunction and subsequent infertility. Furthermore, decreased ovarian estrogen secretion increases the incidence of other diseases, including Alzheimer's, cardiovascular diseases, autoimmune diseases, metabolic syndrome, and gynecological malignancies [3]. The World Health Organization (WHO) classifies POF as hypogonadotropic hypogonadism [4]. Various factors contribute to POF, such as genetic predispositions, autoimmune disorders, or prior anti-cancer treatments (including surgery, radiotherapy, or chemotherapy) [5]. Moreover, long-term exposure to gonadotoxic chemotherapy is increasingly recognized as a significant cause, particularly given the rising incidence of cancer among primates [6]. Approximately 60-80% of chemotherapy patients may experience POF [7].

Cyclophosphamide (CP) is a widely used drug in clinical practice, especially for cancer treatment also exhibits significant reproductive toxicity. CP accelerates the maturation of ovarian follicles, leading to a depletion of the follicular reserve and ultimately resulting in ovarian failure or even POF [8,9]. Gonadotropin and steroid hormones are crucial regulators of folliculogenesis in all mammals. Disrupted endocrine system regulation impairs follicular growth, and follicular storage, contributing to ovarian abnormalities, such as POF. Various researches suggested that fewer primordial and early antral follicles may result in decreased amounts of anti-mullerian hormone (AMH) in the blood. Along with FSH and E2, AMH is a good predictor of POF and represents follicular storage [10]. Following elderly, this tissue atrophies due to ovarian failure and decline in E2. Estradiol promotes the development of free and bound ribosomes, mitochondria, Golgi, and primary lysosomes in glandular cells and likely

in uterine stromal cells. Biochemically, these organelles are essential for protein matrix production, energy provision, and the synthesis of varied enzymes. The form and activity of a functional endometrium reflect the pattern of ovarian hormone secretion. Normally, the glandular cells are typically cuboidal or columnar; therefore, a decrease in E2 leads to the flattening of this epithelium [11]. Oxidative stress has been proposed as a key factor in apoptosis within the reproductive system [12]. Elevated levels of reactive oxygen species (ROS) inhibit follicle development in antral follicles, while antioxidants such as N-acetyl cysteine, can restore ROS and protect ovaries from damage caused by free oxygen radicals [13]. Although the production of ROS over extended periods may have cumulative effects, increasing the risk of ovarian diseases [14]. Despite being the most common treatment for POF, hormone replacement therapy (HRT) has not been shown to restore ovarian function or fertility and carries potential risks of endometrial and breast cancer [11]. Furthermore, multiple attempts at ovarian stimulation are typically unsuccessful. Consequently, a POF diagnosis often leads to significant physical and emotional distress for animals, highlighting the urgent need for novel and effective treatments [15].

Transplanting stem cells has become a viable strategy for restoring compromised ovarian function. Therefore, this study aimed to investigate the therapeutic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) in restoring ovarian function in a rat model of CP-induced POF.

Material and Methods

Ethical Approval

All ethical considerations for the studies on animals were considered carefully and the experimental protocol was approved by the Ethics Committee for research on laboratory animals at Ain Shams University (Ethics Code # ASU-SCI-ZOOL/2023/5/4).

Culture and Isolation of BM-MSCs

Adult male albino rats with average weight of 100-200 g were used for the isolation of BM-MSCs. The tibiae and femurs were flushed with Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) in order to extract bone marrow. Using density gradient Ficoll/Paque (Sigma Aldrich, USA), nucleated cells were separated and subsequently suspended in a full culture medium that contained 1% penicillin-streptomycin (Gibco, USA). The cells were kept in a humidified environment with 5% CO_2 at 37°C. The cultures were washed with phosphate buffer saline (PBS; Lonza, Switzerland) when they reached 80% confluence, and the cells were separated using 0.25% trypsin and 1 mM EDTA (Gibco, USA) for five minutes at 37°C. Following centrifugation, the cells were resuspended in media supplemented with serum. Flow cytometry analysis was employed to immunophenotype the BM-MSCs by detecting the expression of positive indicators include CD90 and CD73, whereas negative markers include the lack of CD45 and CD34 [16].

Experimental Design

A total of 30 healthy adult female Wistar albino rats, weighing between 180 and 190 g, were used in this study. The rats were obtained from the Animal House of the National Research Centre, Egypt. They were housed in a stainless-steel cage and maintained in the animal facility for one week prior to study commencement under a 12/12-h light/dark cycle. Water and a standard diet were given *ad-libitum*. Following a week, the rats were divided into two groups at random: the POF group (n=20) and the normal control group (n=10). Rats were given an intraperitoneal injection of 200 mg/kg CP (Endoxan, Germany) on day 1 and then 8 mg/kg/day for the next 14 days to induce POF [17]. After 14 days, the POF group was randomly divided into two subgroups (n=10/each): POF and POF+BM-MSCs in order to evaluate the impact of BM-MSCs on CP-induced POF. BM-MSCs were adiminstered to the rats via a single intravenous injection into the tail vein (3x106 cells in a 200 µL), and the rats were observed for 4 weeks. The control group did not receive any treatment. Blood samples were drawn from the orbital plexus after the animals were anesthetized with pentobarbital sodium at the end of the experiment. Serum was separated for the subsequent hormonal analysis. The ovaries were excised for biochemical and histopathological analysis and the uterus was removed for histopathological analysis.

Hormonal Assay

The following hormones were measured in rat serum using enzyme-linked immunosorbent assay (ELISA) kits; Follicle-stimulating hormone (FSH, SunLong Biotech Co., Ltd, China, Cat. No: SL0286Ra), luteinizing hormone (LH, SunLong Biotech Co., Ltd, China, Cat. No: SL1093_1Ra), prolactin (PRL, SunLong Biotech Co., Ltd, China, Cat. No: SL0598Ra), progesterone (P4, ALPCO, USA, Cat. No: 55-PROMS-E01), estradiol (E2, SunLong Biotech Co., Ltd, China, Cat. No: SL0268Ra), and anti-Müllerian hormone (AMH, SunLong Biotech Co., Ltd, China, Cat. No: SL0504Ra) were measured in the serum of rats using enzyme-linked immunosorbent assay (ELISA) kits. Thyroid -stimulating hormone (TSH, ALPCO, USA, Cat. No: 55-TSHRT-E01), free triiodothyronine (FT3, ALPCO, USA, Cat. No: MBS260625), and free thyroxine (FT4, ALPCO, USA, Cat. No: SL0295Ra) were similarly determined.

Biochemical Assay

Ovarian tissues were prepared for biochemical analysis

to determine the concentrations of antioxidant enzymes and oxidative stress markers. Briefly, the excised ovaries were washed with ice-cold phosphate-buffered saline (PBS) and mechanically homogenized in 10 volumes (1:10 weight/volume) of ice-cold PBS. The resulting homogenates were centrifuged at 16.000 × g for 10 min at 4°C, and the supernatants were collected for further analysis. Total protein concentration in the ovarian homogenates was determined using the Bradford assay according to the manufacturer's instructions, with bovine serum albumin (BSA) used as the standard. The levels of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were estimated using specific ELISA kits ((RayBiotech, GA, MyBiosource, USA; Cat. No: MBS036924, MBS732529 and MBS701908 respectively), with activities expressed as Units per milligram of protein (U/mg protein). Malondialdehyde (MDA) levels, serving as an indicator of lipid peroxidation, were assessed using a colorimetric assay kit (Elabscience, USA, Cat. No: E-BC-Ko25-S) and normalized to the protein content, expressed as nanomoles per milligram of protein (nmol/mg protein).

Histopathological Examinations

The collected ovarian and uterine tissue samples from rats in the various groups were preserved by fixation in 10% formalin saline for 24 h. The samples were then washed in distilled water, dehydrated through a series of graded ethanol solutions, cleared in xylene and embedded in paraffin wax at 56°C in a hot air oven for 24 h. Paraffin wax tissue blocks were prepared and sectioned at 4 µm using a sledge microtome. For regular light microscopy examination, the acquired tissue sections were deparaffinized, and stained with hematoxylin and eosin stain (H&E) [18].

Statistical Analysis

The mean ± standard deviation (S.D.) is used to display all values. The Statistical Processor System Support (SPSS) program (version 18.0; SPSS Inc., Chicago, IL, USA) was used to conduct statistical analysis. One-way analysis of variance (ANOVA) and the Tukey's honestly significant difference (HSD) post hoc test were used to evaluate the data.

Results

Flow cytometric analysis was performed to identify specific cell surface markers of BM-MSCs. The findings are consistent with the anticipated immunophenotype of BM-MSCs *(Fig. 1)*, revealing the cells to be negative for hematopoietic (CD34, CD45) markers and strongly positive for mesenchymal stromal cell markers (CD73, CD90).

The present study demonstrated a significant reduction in serum levels of E2 (52.27±2.94 pg/mL), P4 (8.26±1.80 ng/mL), and AMH (0.97±0.07 ng/mL) in rats within

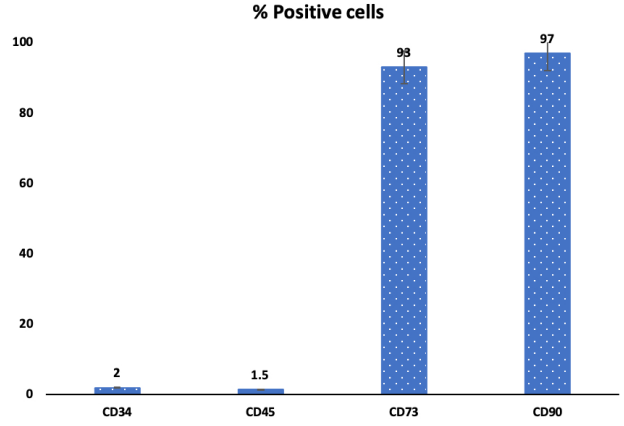

Fig 1. The percentage of positive cells, indicating the proportion of the BM-MSCs population expressing each marker. The x-axis lists the different CD markers analyzed: CD34, CD45, CD73, and CD90.The error bars represent the standard error of the mean, indicating the variability within the measurements. The error bars are relatively small for CD73 and CD90, suggesting consistent expression of these markers across the analyzed samples

the POF group (P<0.001). Conversely, a significant elevation (P<0.001) in serum levels of FSH (6.94±0.50 IU/L), LH (5.37±0.73 IU/L), and PRL (0.98±0.22 ng/mL) were observed in the POF group comparing to the control group *(Fig. 2)*. In contrast, treatment with BM-MSCs transplantation resulted in a significant increase in serum E2 (79.06±5.76 pg/mL, P<0.001), P4 (13.07±1.23 ng/mL, P<0.01), and AMH (1.98±0.11 ng/mL, P<0.001). Concurrently, serum levels of FSH (3.97±0.74 IU/L, P<0.01), LH (3.57±0.30 IU/L, P<0.01), and PRL (0.72±0.07 ng/mL, P<0.05) were significantly reduced compared to the POF group. However, no significant differences were observed in these parameters when comparing rats in the control group and the POF+BM-MSCs group *(Fig. 2)*.

Rats in the POF group exhibited a significant reduction in serum levels of FT3 (1.50±0.33 pg/mL, P<0.001) and FT4 (0.80±0.04 pg/mL, P<0.01). Conversely, serum level of TSH was significantly elevated (3.67±0.51 µIU/mL, P<0.01) compared to control group *(Fig. 3)*. In contrast, treatment with BM-MSCs transplantation resulted in a significant increase (P<0.01) of FT3 (2.42±0.37 pg/mL) and FT4 (1.62±0.48 pg/mL). Concurrently, TSH was significantly decreased (2.74 µIu/mL) in contrast with the POF group (P<0.05). However, no significant notable variations were observed in these parameters in POF+BM-MSCs group comparing to the control group *(Fig. 3)*.

In rats within the POF group, significant reduction (P<0.001) was observed in the concentrations of SOD (74.48±3.62 U/gm tisssue), GPx (35.22±4.46 u/gm protein), and CAT (134.70±2.78 u/gm protein). Conversely, MDA levels were significantly elevated (2.81±0.35 nmol/gm protein, P<0.01) compared to the control group *(Fig. 4)*. In contrast, treatment with BM-MSCs transplantation led to

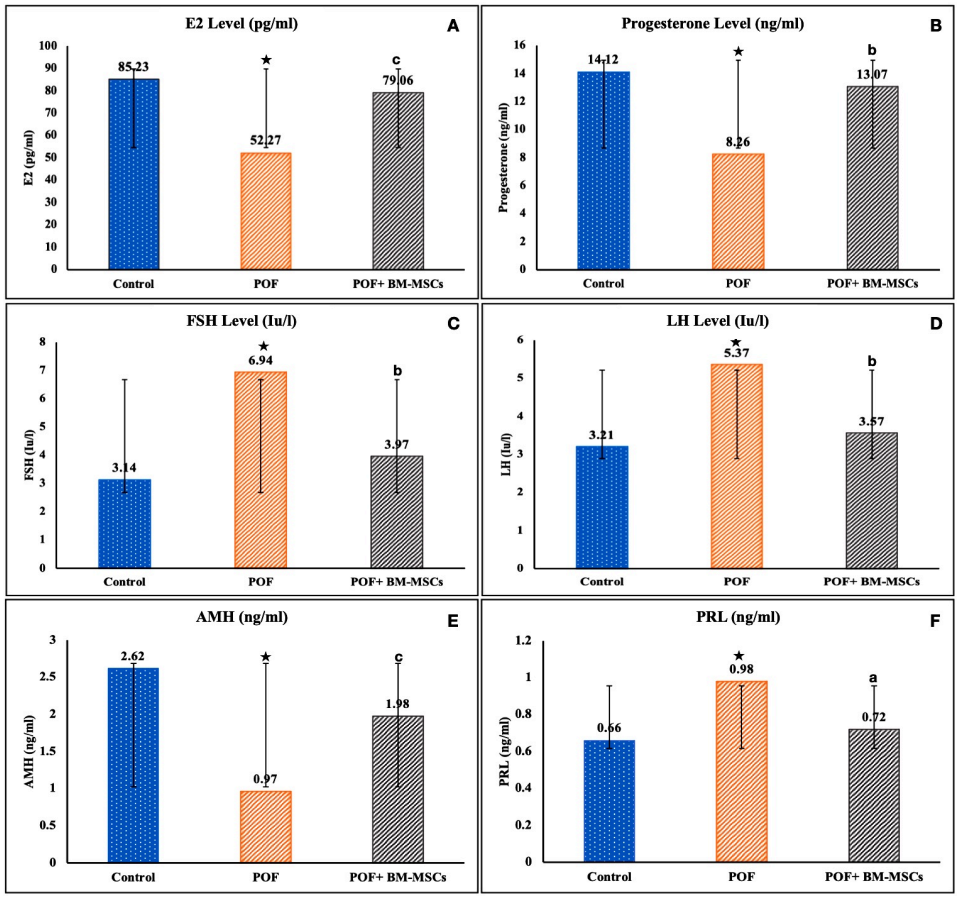

Fig 2. Effect of CP administration and subsequent treatment with BM-MSCs transplantation on serum E2, P4, FSH, LH, AMH and PRL levels in female albino rats. * is significantly different than control at P<0.001. a, b, c are significantly different than POF at P<0.05, P<0.01 and P<0.001 respectively. POF: premature ovarian failure, BM-MSCs: bone marrow mesenchymal stem cells

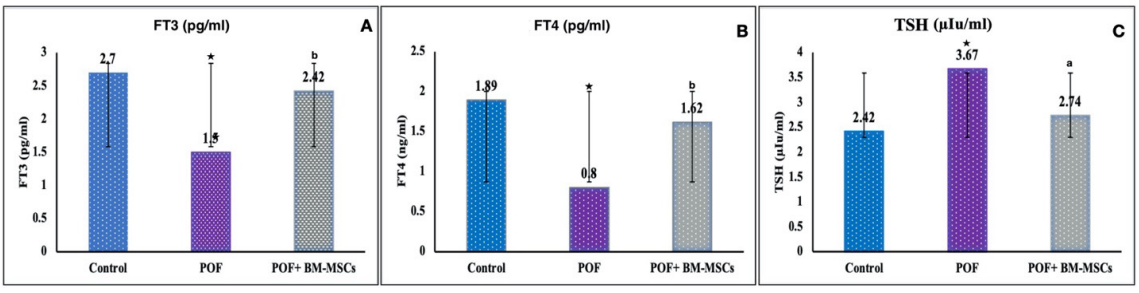

Fig 3. Effect of CP administration and subsequent treatment with BM-MSCs transplantation on serum FT3, FT4 and TSH levels in female albino rats. * and ** are significantly different than control at P<0.01 and P<0.001 respectively. a, b are significantly different than POF at P<0.05 and P<0.01 respectively. POF: premature ovarian failure, BM-MSCs: bone marrow mesenchymal stem cells

a significant increase of SOD (93.46±4.25 U/gm protein, P<0.001), GPx (47.07±3.06 u/gm protein, P<0.05), and CAT (183.48±2.67 u/gm protein, P<0.001). Concurrently, MDA levels were significantly decreased with (1.98±0.31 nmol/gm protein) compared to the POF group (P<0.01).

In the histological examination, control ovary exhibited various stages of follicular maturation and graafian follicles accompanied by corpus luteum and interstitial stromal cells consistent with normal histological structures *(Fig. 5-A,B)*. Conversely, the ovaries of POF rats displayed degeneration and nuclear pyknosis within the theca interna cell layer of the mature follicles in the ovarian tissue. The stromal cells situated between the follicles were increased, potentially due to impaired ovulation, and multiple corpus luteum were observed *(Fig. 5-C,D)*. However, treatment with BM-MSCs resulted in the restoration of normal histological structures, characterized by the presence of Graafian follicles, corpus lutea and various stages of follicular maturation *(Fig. 5-E,F)*. It was found that CP administration resulted in a notable reduction in the number of ovarian follicles *(Fig. 5-C,D)*.

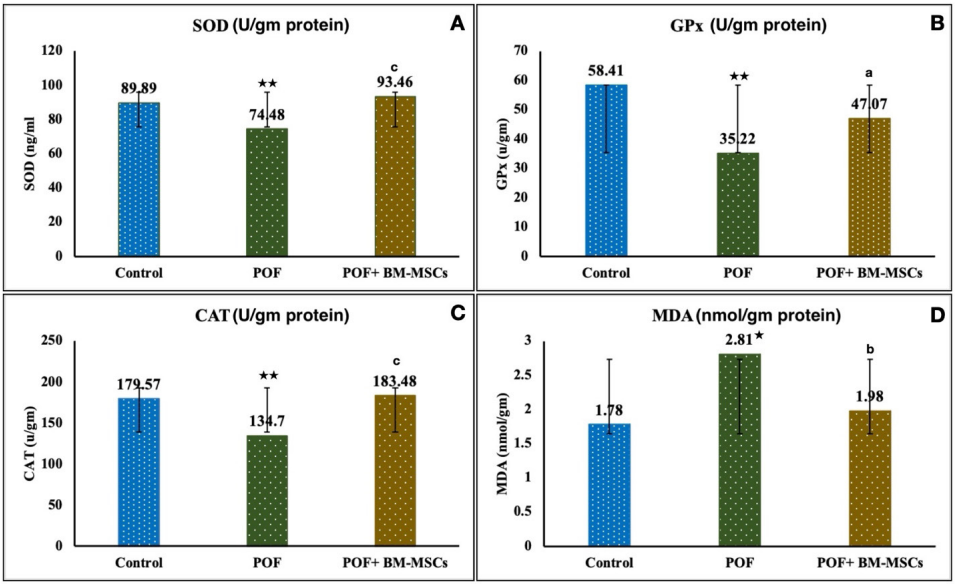

Fig 4. Effect of CP administration and subsequent treatment with BM-MSCs transplantation on SOD, GPx, CAT and MDA levels in ovarian tissue of female albino rats. * and ** are significantly different than control at P<0.01 and P<0.001 respectively. a, b are significantly different than POF at P<0.05, P<0.01, and P<0.001 respectively. POF: premature ovarian failure, BM-MSCs: bone marrow mesenchymal stem cells

Regarding the uterine structure, the histological structure of the mucosa was normal in control rats including the lining epithelium and the underlying lamina propria containing glandular structure, as well as the submucosa, muscularis and serosa *(Fig. 6-A,B)*. In contrast, the uteri of POF rats displayed a flattened mucosal epithelium with atrophy of the glandular structures in the underlying lamina propria with degeneration of the uterine glandular lining epithelial cells within the lamina propria mucosal layer *(Fig. 6-C,D)*. Following treatment with BM-MSCs, degeneration was observed in the uterine glandular lining epithelial cells within the lamina propria and mucosa, and atrophy was noted the in uterine glands within lamina propria *(Fig. 6-E-F)*. Moreover, CP administration led to flattened mucosal epithelium with atrophy of the glandular structure in the underlying lamina propria and degeneration in the uterine glandular lining epithelial cells within the lamina propria mucosal layer *(Fig. 6-C,D)*. However, BM-MSCs transplantation did not exhibit a significant effect on uterine tissue.

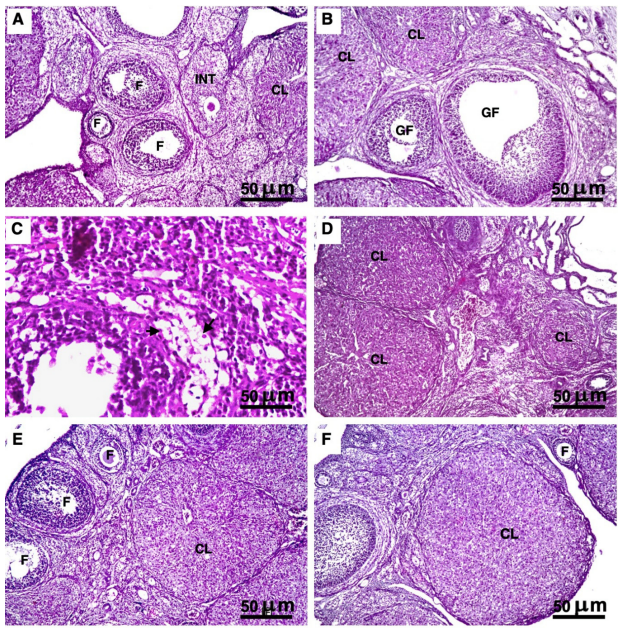

Fig 5. Histological examination of ovaries of different groups. (A) Photomicrograph of control ovary showing different stage of follicles (F) with corpus luteum (CL) and interstitial stromal cells (INT) in normal histological structure (H&E, ×16). (B) The graafian follicles (GF) and corpus luteum (CL) in normal histological structure (H&E, ×16). (C, D) Photomicrograph of ovary of POF rats showing degeneration and nuclear pyknosis in the theca interna cell layer of the mature follicles in ovarian tissue (arrows) and multiple corpus luteum (CL) with stromal cells in between the follicles are increased due to weak ovulation (H&E, ×16). (E, F) Photomicrograph of ovary of BM-MSCs treated rats showing normal histological structure of the graafian follicles (GF) and corpus luteum (CL) and different stages of follicular maturation (F) (H&E, ×16)

Discussion

Stem cells, with their remarkable ability to self-renew and differentiate into various specialized cell types, hold immense potential in regenerative medicine. They offer hope for treating a wide array of diseases and injuries. Several studies were exploring stem cells use to repair damaged tissues, grow replacement organs, and develop new drug therapies [19].

In this study BM-MSCs were isolated to test the possibility that they could increase fertility and restore ovarian function. Flow cytometric analysis was performed to identify specific cell surface markers of BM-MSCs. The findings are consistent with the anticipated immunophenotype of BM-MSCs. This specific pattern

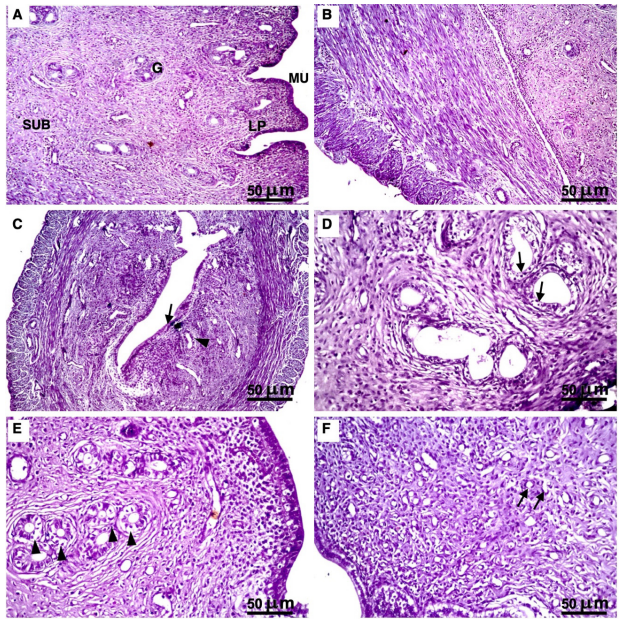

Fig 6. Histological examination of uterus of different groups. (A, B) Photomicrograph of uterus of control group showing normal mucosal (MU) histology, including lining epithelium and underlying lamina propria (LP) with glandular structure (G) and submucosa (SUB) (H&E, ×16). (C) Photomicrograph of uterus of POF rats showing flattened mucosal epithelium (FMU) with atrophy of uterine glands (arrow head) (H&E, ×16) and (D) showing degeneration (arrow) in uterine glandular lining epithelial cells in lamina propria mucosal layer (H&E × 40). (E) Photomicrograph of uterus of BM-MSCs treated rats showing degeneration (arrow head) in uterine glandular lining epithelial cells in lamina propria and mucosa (H&E × 40) and (F) showing atrophy (arrow) in uterine gland in lamina propria (H&E × 40)

of marker expression serves as a crucial criterion for the definition and characterization of BM-MSC populations isolated using flow cytometry as reported previously by Abo-Aziza et al.[20].

The reduction in the levels of E2, P4 and AMH and the elevation of FSH, LH, and PRL in serum the POF group were align with previous studies [21] that reported increased serum FSH and LH following CP administration. Hence, CP-induced POF is characterized by severe follicular depletion and a subsequent loss of regular cyclicity, often resulting in persistent diestrus. This hormonal profile reflects a significant ovarian toxic effect, evidenced by the substantial decrease in serum P4, E2, and AMH, coupled with a notable decline in the number of follicles, ultimately leading to POF. Furthermore, several studies have shown that chemotherapeutic drugs can induce POF across various species, including mice, rats, rabbits, and humans [22,23]. A potential mechanism for the observed reduction in serum E2 and P4 levels involves alkylating agents. Cyclophosphamide (CP), recognized as one of the most ovotoxic chemotherapy drugs, creates DNA crosslinks, subsequently inducing DNA breaks and ultimately triggering apoptosis [24]. CP causes ovarian toxicity due to a compromised antioxidant defense system and an overabundance of free radicals, leading to apoptosis in granulosa cells (GCs) within the follicles and was associated with the significant decreased in E2 and P4 [9]. This process may involve alteration in endogenous antioxidant enzymes within healthy ovarian tissues, as indicated in the present study. Another contributing factor could be the strong toxic effects CP exerts on the gonads, with its medical applications resulting in POF. CP use contributes significantly to iatrogenic consequences and can result in diseases including amenorrhea, infertility, menstrual abnormalities, and diminished libido. POF hinders follicular stage development and may lead to interstitial fibrosis, necrosis, and endocrine alterations that can increase blood levels of LH and FSH while decreasing levels of E2 and P4 [25,26].

Female ovarian granulosa cells generate AMH, one of the transforming growth factors β-super family. Serum AMH levels fluctuate according to developmental stage, starting during puberty and falling off throughout aging. AMH has shown considerable value as an indication of ovarian reserve function. As ovarian reserve function declines with age, changes in AMH levels manifest earlier than changes in FSH, E2, and antral follicle count. Moreover, pregnancy, hormonal contraceptives, or the menstrual cycle have no effect on its concentration [27], establishing it as the most accurate biomarker of ovarian aging. Additionally, a significant elevation in serum FSH and LH was observed following CP administration. This increase may be attributed to the substantial decrease in E2 levels, which disrupts the negative feedback mechanism, leading to the stimulation of GnRH from the hypothalamus and consequently a significant rise in FSH and LH secretion from the anterior pituitary [28,29]. In contrast, treatment with BM-MSCs transplantation resulted in a significant increase in serum E2, P4, and AMH. Concurrently, the significant elevation of FSH and LH in the POF group followed by their significant reduction after BM-MSC treatment reflects a stable shift in the endocrine profile rather than transient fluctuations. The trigger of POF is multifaceted and can be attributed to various factors, including genetic defects, autoimmune reactions, chemotherapy, radiotherapy, and surgery. Stem cells, as a multipotent cell type with the capacity for self-renewal and multilineage differentiation, have been identified as a promising tool in tissue engineering for regenerative medicine [30,31]. Their potential as a treatment provides a fresh approach to protecting or repairing impaired ovarian function in primates undergoing radiation or chemotherapy. The following are some theories as to how stem cells in POF restore the impaired ovarian function: The process by which stem cells migrate into damaged ovarian tissue and differentiate into cells that resemble ovarian tissue, especially granulosa cells, which are vital

parts of the ovarian microenvironment and are essential for controlling ovarian physiology, including luteal regression and ovulation [32]. The homing and residence of stem cells within ovarian tissue, where they contribute to improving damaged ovarian niches among paracrine effect by secreting various growth factors, cytokines, angiogenic factors, and extracellular matrix proteins [33]. Moreover, the effective alleviation of chemotherapy-induced inflammatory reactions in ovarian tissue through the secretion of anti-inflammatory substances following stem cell transplantation [34]. Moreover, MSCs may lower GC apoptosis by affecting G-protein coupled receptor protein signalling and MAPK pathways, both of which are essential for follicle and oocyte formation [35]. These mechanisms support the therapeutic benefit of stem cell therapy for improving ovarian function in individuals with POF. Elevated FSH levels can accelerate follicle recruitment and deplete the follicular pool. Deceased E2 and FSH levels could result from increased apoptosis in the POF group. Stem cell transplantation increased E2 secretion and prevented granulosa cell death, subsequently, leading to downregulation of FSH and LH secretion from the pituitary gland and the consequent inhibition of follicular recruitment [36].

Chemotherapy stands as one of the most effective systemic therapies for cancers. In cancer patients, thyroid function is considered susceptible to chemotherapy due to the active hypothalamic-pituitary axis and the systemic nature of the treatment. The impact of chemotherapy on thyroid function was thought to be a side effect that mostly showed up as hypothyroidism. Thyroid hypofunction was thought to be connected with the suppression of hepatic thyroglobulin secretion and an increase in antithyroid antibodies, specifically anti-thyroid peroxidase (TPO) and antithyroglobulin, induced by chemotherapy [37]. It is hypothesized that endocrine cells with compromised mitochondria exhibit elevated oxidative stress and/or decreased adenosine triphosphate synthesis, along with distention and vacuolization of the endoplasmic reticulum cisternae within thyroid follicles, may result in the inability to synthesize and/or secrete hormones during cyclophosphamide chemotherapy [37]. In contrast, treatment with BM-MSCs transplantation resulted in a significant increase of FT3 and FT4. Concurrently, TSH was significantly decreased in contrast with the POF group. However, no significant notable variations were observed in these parameters in POF+BM-MSCs group comparing to the control group.

In rats within the POF group, significant reduction was observed in the concentrations of SOD, GPx, and CAT. Conversely, MDA levels were significantly elevated. The toxicity of CP exacerbated lipid peroxidation in ovarian tissue. MDA, a by-product of lipid peroxidation, acts as oxidative stress indicator. Furthermore, Cyclophosphamide-induced ovarian damage resulted in decreased concentrations of SOD, GPx, and CAT. Oxidative stress inhibited both nuclear and cytoplasmic maturation of oocytes, and triggering apoptosis led to ovarian failure [38]. In contrast, treatment with BM-MSCs transplantation led to a significant increase of SOD, GPx, and CAT. Concurrently, MDA levels were significantly decreased with compared to the POF group. Stem cells transplantation presents an ideal potential treatment for repairing cytotoxic effect of CP and serves also a powerful tool in restoring fertility and the potential of pregnancy, consequences that could be linked to stem cells' paracrine, homing, and differentiation [39,40]. Control ovary exhibited various stages of follicular maturation and graafian follicles, accompanied by corpus luteum and interstitial stromal cells consistent with normal histological structures. Conversely, the ovaries of POF rats displayed degeneration and nuclear pyknosis within the theca interna cell layer of the mature follicles in the ovarian tissue. The stromal cells situated between the follicles were increased, potentially due to impaired ovulation, and multiple corpus luteum were observed. However, treatment with BM-MSCs resulted in the restoration of normal histological structures, characterized by the presence of Graafian follicles, corpus lutea and various stages of follicular maturation.

Regarding the uterine structure in this study, the histological structure of the mucosa was normal in control rats including the lining epithelium and the underlying lamina propria containing glandular structure, as well as the submucosa, muscularis and serosa. In contrast, the uteri of POF rats displayed a flattened mucosal epithelium with atrophy of the glandular structures in the underlying lamina propria with degeneration of the uterine glandular lining epithelial cells within the lamina propria mucosal layer. Following treatment with BM-MSCs, degeneration was observed in the uterine glandular lining epithelial cells within the lamina propria and mucosa, and atrophy was noted the in uterine glands within lamina propria.

This study demonstrated a significant decrease of the count of Graafian follicles and other follicles in female rats within the POF group, while the corpus luteum count was significantly increased. These results are consistent with the observation of Zhang et al.[41] that CP increases ovarian follicle maturation, decreases follicular reserve, and ultimately causes ovarian failure or even POF. A potential explanation for these observations lies in the critical role of ovarian granulosa cells (GCs) are the primary stroma cells within the ovary, surrounding the oocyte and playing a key role in folliculogenesis [42]. Given their capacity to secrete growth factors and provide hormonal support, GCs are essential for oocyte growth and survival. The main cause

of POF and follicular atresia is increased GC apoptosis that carried out by chemotherapy [43]. In this study, it was found that CP administration resulted in a notable reduction in the number of ovarian follicles. This decrease in ovarian follicles may be due to the reduction in serum E2 which is necessary for early folliculogenesis. Decreased E2 level inhibits follicular progression, thereby disrupting folliculogenesis. FSH receptors are predominantly expressed on GCs. Elevated FSH level can accelerate the depletion of the follicular pool; thus, increased FSH acts as both a cause and a marker diminished ovary reserve [44]. This study also revealed a significant decrease in serum AMH. AMH has been used as a predictor of the quality of oocytes remaining within the ovaries (ovarian reserve) and has been shown to correlate with antral follicle counts and outcomes of ovarian stimulation. Consequently, AMH was previously considered to be an ideal marker of ovarian reserve due to its exclusively production by GSc. According to research on animals, AMH influences the recruitment of primordial follicles and how developing follicles react to FSH [45].

Furthermore, the oocyte has both isoforms of thyroid hormone receptor mRNA, indicating that thyroid hormone may have a direct effect on the oocyte. Rat preantral follicle growth and ovulated oocyte counts are both stimulated by thyroid hormone. T3 stimulated granulosa cell proliferation and suppressed apoptosis by activating the PI3K/Akt signaling pathway [46]. Hence, the decreased serum T3 observed in this study may have contributed to the reduction in ovarian follicles and increased granulosa cell apoptosis. In contrast, following treatment with BM-MSCs, normal histological structure of the Graafian follicles and corpora lutea, along with various stages of follicular maturation, were observed. These findings are consistent with the study of Jalalie et al.[47], who reported that transplantation of MSCS reduced degenerative changes and increased follicular quantitative parameters in ovarian follicles compared to the CP group. These beneficial effects may be attributed to stem cells' migration into damaged ovarian tissue and subsequent differentiation into cells that resemble ovarian tissue, especially granulosa cells [34].

In this study CP administration led to flattened mucosal epithelium with atrophy of the glandular structure in the underlying lamina propria and degeneration in the uterine glandular lining epithelial cells within the lamina propria mucosal layer. These results coincide with the observation of Chen et al.[48], who reported that CP administration caused damage to the ovary, potentially leading to primary ovarian insufficiency. These results may be due to CP-induced significant decrease in E2 and FSH, resulting in defects in folliculogenesis and subsequent atrophy in the uterus. However, BM-MSCs transplantation did not exhibit a significant effect on uterine tissue in this study. The lack of significant uterine tissue improvement after BM-MSC transplantation in this study could stem from the therapeutic regimen's duration and delivery route. A single systemic dose might have been insufficient for the uterus, potentially requiring repeated administrations or a longer observation period for noticeable effects. Furthermore, systemic delivery might have resulted in limited MSC homing to the uterus compared to the ovaries, where damage signals were stronger. A more direct delivery route targeting the uterus could be necessary to achieve a therapeutic concentration of BM-MSCs in that specific tissue. The uterine microenvironment might also necessitate a more sustained or different stimulus from BM-MSCs than the ovaries.

In conclusion, the present study offers novel insights into the significant clinical potential of BM-MSCs therapy in premature ovarian failure by ameliorating the disrupted endocrine secretion system, mitigating ovarian toxicity through the improvement of tissue oxidative stress marker levels, modulating thyroid hormones and TSH, and alleviating histological damage in ovarian tissue. Furthermore, future studies investigating the therapeutic potential of BM-MSCs for POF-related uterine issues might consider exploring different dosages, repeated administrations, alternative routes of injection (potentially local), and longer follow-up periods to better understand their effects on this tissue.

Declarations

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