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Mesenchymal stem cells promote the repair of chemotherapy-induced premature ovarian failure in female rats

Heba E. Sherif [1*], Yakout A. El- Senosi[1], Samy A. Hussein[1], Ehab M. Elzoghby[2], Mohamed A. Sobh[3], Sara M. Farrag[4], Basma H. Othman[4]

[1] *Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Benha University, Egypt*

[2] *Department of Histology, Faculty of Veterinary Medicine, Benha University, Egypt*

[3] *Urology and Nephrology Center, Faculty of Medicine, Mansoura University, Egypt*

[4] *Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Egypt*

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ABSTRACT

Chemotherapeutic drugs, particularly alkylating cytotoxics such as cyclophosphamide (CYP) play a great job in inducing premature ovarian failure (POF). Hormone replacement therapy (HRT) is a widely used medication to improve hormone secretion. However, long-term HRT increases the risk of breast cancer and cardiovascular disease is concerning. Therefore, there is an urgent need to develop a safe and effective treatment for POF. Bone Marrow-(BM- MSCs) and Umbilical Cord -(UC- MSCs) derived stem cells (SCs) were isolated and identified from healthy rats. For POF induction, CYP (200mg/Kg b.wt) injected (i.p) into CYP, CYP+BMSCs, and CYP+UCMSCs followed by another dose (10mg/ b.wt, i.p) after one week. The normal control (NC) group were injected with saline. After two weeks of POF induction MSCs (1×10^6 cells/ml) of media were transplanted into POF rats through tail-vein. The effects of MSCs transplantation to POF were evaluated through sex hormones, iron, and complete blood count (CBC) analysis. POF-induced rats showed a significant increase in serum FSH and LH, with marked decrease in anti-Mullerian hormone (AMH) and estradiol (E2). Obvious increase in serum iron level observed in CYP group compared to the NC. Moreover, POF-induced rats displayed leucopenia, erythrocytopenia, and thrombocytopenia with a significant reduction in Hb, PCV, MCV, MCH, and MCHC compared to the NC. Administration of MSCs to CYP-injured rats significantly improved female reproductive hormones disturbances, iron, and hemogram. It was suggested that MSCs was a potential approach for improving sex hormones, iron, and hematological alterations, and potentially promoting the restoration of CYP-induced POF.

1. INTRODUCTION

Premature ovarian failure (POF), also known as premature menopause, is a common condition, affecting 1–2% of women younger than 40 years of age and 0.1% of women younger than 30 years of age (Coulam *et al*. 1986). It is distinguished by high expression of follicle-stimulating hormone (FSH), low estradiol (E2) expression, and inadequate follicular development and termination of ovarian function in women under the age of 40 (Jankowska, 2017). It is believed that the formation and progression of POF are closely associated with many risk factors including hormonal, immune, metabolic, environmental, and genetic factors. Approximately 74%–90% of POF cases have unknown causes (Little and Ward, 2014). Tissue engineering and regenerative medicine have recently gotten great attention and some impressive advancement. This technique aims to repair damaged tissues and organs or to preserve and enhance their function. Recent advances in stem cell technology provide new hope for people suffering from illnesses and disorders that have not been completely treated. In animal experiments, mesenchymal stem cell (MSCs) transplantation might be a promising treatment for POF because of its safety and productivity (Zivari-Ghader et al., 2022; Guo al., 2023). These benefits may be linked to improving the sexual cycle disorder and restoring ovarian function. Stem cell-based therapy is an important branch of regenerative medicine with the ultimate goal of enhancing the body repair machinery via stimulation, modulation, and regulation of the endogenous stem cell population replenishing the cell pool toward tissue homeostasis and regeneration (Hoang et al., 2022). Since the stem cell definition was introduced with their unique self-renewal and differentiation properties, they have been subjected to numerous basic research and clinical studies and are defined as potential therapeutic agents. To achieve the targets of regenerative medicine with tissue regeneration and cellular replacement different types of stem cells have been used, including blood, placenta, umbilical cord (UC), adipose tissue (AD), bone marrow (BM), and menstrual fluid (Gao et al., 2022; Huang et al., 2022). MSCs transplantation improves ovarian injury and restores ovarian function in a chemotherapy-induced POF model (Chen et al. 2021; Ghazal et al., 2023). Today, 5% of cancer patients are under the age of 50, and female survivors of aggressive treatment procedures are more likely to have ovarian infertility issues

Correspondence to: Heba.sherif2705@gmail.com

(Siegel et al., 2016). The degree of ovarian damage and risk of infertility are determined by the amount and kind of chemotherapeutic drug used, as well as the woman's age at the time of treatment (Roness et al., 2014). One of the most commonly used chemotherapeutic treatments for cancer, the alkylating agent, cyclophosphamide (CYP), has a high risk of ovarian failure (İlgen et al., 2023). CYP is extensively used in a range of malignant tumors and immunological disorders as well as organ transplantation (Khazaei et al., 2020; Sailor et al., 2021). Hemotoxicity is a common adverse effect of CYP during chemotherapy. CYP-treated mice show leucopenia, erythrocytopenia, and thrombocytopenia as well as a significant decrease in hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC) as compared to control group (Kausar and More, 2019; Khazaei et al., 2020). High iron levels have been linked to both a common illness and an elevated risk for numerous malignancies, which is further exacerbated by cancer treatment (Miya et al., 2018). Ferroptosis of granulosa cells and oocyte dysmaturity may induce iron excess resulting in infertility, and have a direct role in female gonadal function (Ni et al., 2022; Holzer et al., 2023). We hypothesized that the symptoms of iron poisoning are similar to the side effects of chemotherapy. Thus, the current study investigated the relationship between the hematologic parameters, iron level, and hormonal profile in female rats treated with MSCs derived from bone marrow or umbilical cord suffering from infertility due to POF damage caused by CYP.

2. MATERIAL AND METHODS

2.1. Experimental Animals

The research employed eight-week-old female Sprague-Dawley rats with a pathogen-free (SPF) grade and weighing 180-220 g. The rats were inbred in the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Egypt. The rats were kept under normal laboratory settings (temperature: 25 ± 2°C, humidity: 60 ± 5%, 12-h dark/light cycle) and given a conventional unrestricted diet and water supply. All the experimental procedures complied with the Institutional Animal Care and Use Committee according to the institutional guidelines and national animal welfare with the principles of the Declaration of Helsinki (Approval No. BUFVTM- 11-10-2023)

2.2. Animal Model Establishment and Experimental Design

Cyclophosphamide (CYP) was purchased from (Cycram, KUP-EIMC Pharmaceuticals Co., Korea).To develop the induced POF in a rat model, CYP (200mg/Kg b.wt) was injected intraperitoneally (i.p) followed by another activation dose of CYP (10mg/ b.wt, i.p) after one week (Abogresha et al., 2021). After two weeks MSCs (1×10^6 cells/mL) were transplanted into POF-induced rats through tail-vein (Wang et al., 2020). The POF group (n = 30) was further divided into three groups (n = 10 each); The CYP group was injected with CYP only and kept as a positive control group, The CYP- BM group was injected with CYP and BM-MSCs treatment one week after the induction, CYP-UC group was injected with CYP and UC-MSCs as in BM-MSCs group. The rats in the normal control (NC) group (n = 10) were injected i.p with saline. Each group was euthanized one and two weeks after MSCs treatment.

2.3. Rat BM-MSCs Isolation

BM-MSCs were obtained from the tibias and femurs of healthy Sprague-Dawley (Sobh, 2014) rats aged 5 weeks. BM-MSCs were cultured in low glucose Dulbecco's modified Eagle's medium (L-DMEM) (Capricorn Scientific, Germany) supplemented with 10% fetal bovine serum (Thermo Fisher, Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were seeded at 1×10^6 cells/mL density into culture flasks and grown in a humidified incubator at 37°C and 5% CO2. The medium was replaced after 48 h of incubation, and adherent cells were recognized as BM-MSCs, they were used after seven passages when they had a consistent morphological appearance of fibroblast-like long spindles in an orderly arrangement.

2.4. Rat UC-MSCs Isolation

UC-MSCs were collected from pregnant rats (Zhang et al., 2018) and prepared as follows: Before rat birth, UC was removed, resulting in a full-term chord. The UC was manually dissected into 1-2 mm 3 sections and incubated with 0.075% collagenase type II for 30 minutes, followed by 0.125% trypsin for 30 minutes with moderate agitation at 37◦C. UCs were plated at a density of 1×10^6 cells/mL in cell culture flasks containing L-DMEM, supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin at 37◦C in a humidified environment of 5% CO2. After three days, nonadherent cells were eliminated, and after three changes to the media, the first UC-MSC colonies emerged. Passage 2 cells were collected with 0.25% trypsin-EDTA and were separated into single cells for further examination and transplantation.

2.5. MSCs identification, differentiation, and Systemic Transplantation

MSCs surface marker expression was measured by a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) using CD29-PCYNA, CD44-FITC as positive markers, CD34- PCYNA, and TER119-PE as negative markers (all from Biolegend, San Diego, California). MSCs differentiated for osteogenesis by cultivating them in the osteogenic induction medium (Cyagen, Santa Clara, USA) for 14-21 days. Alizarin red staining was used to confirm calcium deposition. To transplant BM-MSCs or UC-MSCs into POF rats, 1×10^6 cells third-passage in 100 µL of PBS was injected into the tail vein (Wang et al., 2020). The control group of rats received a 100 µL injection of PBS.

2.6. Hormone profile

The hormonal profile was examined to validate the diagnosis of CYP-induced infertility. These hormones included anti-Müllerian (AMH), estradiol (E2), follicular stimulating (FSH), and luteinizing hormone (LH). To determine their levels in serum, blood samples were collected from rats' models by heart puncture while anesthetized at sacrifice. Serum was collected by centrifugation at 4000 rpm for 10 minutes. According to the manufacturer's instructions, the hormone concentrations were determined by ELISA kits (SunLong Biotech Co., LTD, Zhejiang, China).

2.7. Determination of Iron concentration

Serum iron was measured on an automated platform (Cobas C311 from Germany). The reaction is done at a pH of <2.0, where iron interacts with an acid and is reduced by ascorbate to ferrous iron.

Divalent iron ions create an iron-colored complex that is detectable at 552 nm (Siedel et al., 1984).

2.8. Measurements of Hematological parameters

Approximately 1 ml of samples was collected in EDTA-containing tubes by cardiac puncture during sacrifice under anesthesia. All blood samples were promptly analyzed for a complete blood count. The "Hematology auto-analyzer Sysmex x100" was used to measure various hematological parameters, including erythrocyte count, leukocyte count, thrombocyte count, hemoglobin concentration (Hb), % hematocrit (HCT), the Packed Cell Volume (PCV), the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and MCH concentration (MCHC).

2.9. Statistical Analysis

Continuous variables were represented as the mean ± standard error means (SEM). The data were examined using the T-test comparing groups and analysis of variance (ANOVA) followed by Bonferroni's post hoc analysis was used for comparisons between groups. The significant difference p value less than 0.05 ($p < 0.05$) was considered statistically significant. The statistical software package SPSS for Windows (Version 20; SPSS Inc., Chicago, IL, USA) was used.

3. RESULTS

3.1. Identification of Mesenchymal stem cells

Figure 1 represents the identification of MSCs. Flow Cytometry (FCM) results indicated that the MSCs exhibited positive mesenchymal progenitor markers with CD44 and CD29 cells, but did not show expression of negative signals with CD34 and TRE119 cells. The purity of the isolated cells exceeded 98%. MSCs may develop into different mesoderm-type cells, including osteoblasts, as seen by the Alizarin Red stain

3.2. MSCs transplantation regulates ovarian sex hormone expression

Serum reproductive hormone levels of all rats in separate groups after one- and two weeks post MSCs therapy were presented in Figure 2. Briefly, CYP causes declines in both AMH and E2 which statistically significantly improved with MSCs therapy approaching those in the NC group. CYP induced greater elevations in both FSH and LH than the NC group, and MSCs therapy resulted in statistically significant improvements comparable to the NC group

3.3. Iron overload regulated after MSCs Transplantation

After one and two weeks of MSCs therapy, the CYP-induced serum iron overload level improved statistically significantly, approaching that of the NC group, as shown in (Figure 3). UC-MSCs respond better than BM-MSCs, particularly after two weeks of therapy ($P = 0.007$)

3.4. Transplantation of MSCs ameliorate hematological alterations induced by CYP

CYP causes hematological toxicity which improved with MSCs therapy and is most noticeable after two weeks of medication. The CYP group had a considerably lower leukocyte count ($P<0.0001$), Hb concentration ($P<0.0001$), HCT% ($P<0.0001$), and thrombocyte count ($P<0.0001$) when compared to the NC group. MSCs therapy ameliorated these decreases, particularly after two weeks ($P<0.0001$). The UC-MSCs group responded better than the BM-MSCs group (particularly in leukocyte count; $P=0.001$) after one week of therapy (Figure 4). Thrombocyte count did not vary. The CYP group had a considerably lower erythrocyte count ($P<0.0001$), and packed cell volume (PCV), and consequently MCV, MCH, and MCHC ($P<0.0001$) when compared to the NC group. MSCs therapy significantly restores the drops in erythrocyte count and PCV index after two weeks ($P<0.0001$). BM-MSCs or UC-MSCs respond similarly to the NC group, particularly after two weeks of therapy (Figure 5)

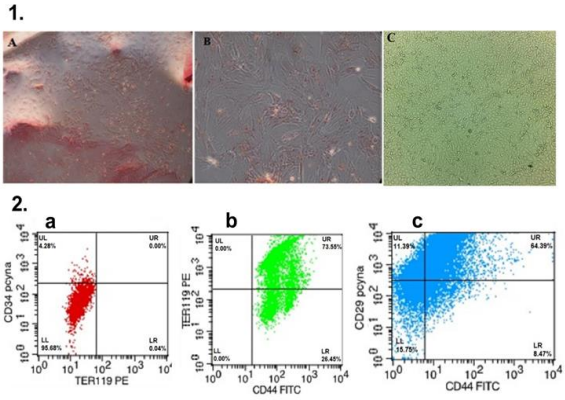

Figure 1: Identification of the isolated and cultured rat mesenchymal stem cells. 1. Alizarin Red stain revealed cellular aggregates (A) in the BM-MSCs cultures and (B) in the UC-MSCs cultures. (C) The isolated MSCs exhibited typical fibroblast-like morphology. Bar: 100µm. 2. FCM findings of MSCs (D). a, the population of negative MSC Markers CD34 and TRE119; b, the population of negative TRE119 and positive CD44 cells and c, the population of positive MSC Markers CD44 and CD29

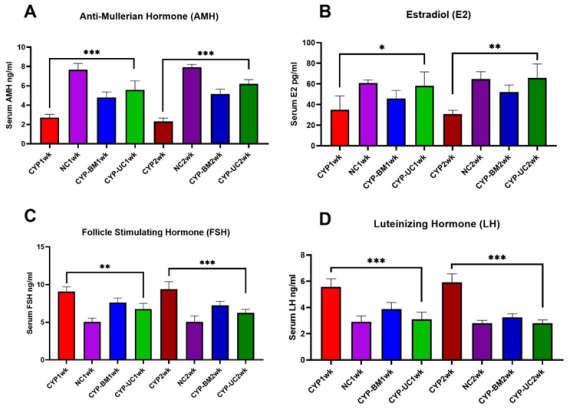

Figure 2: ELISA measurements of hormonal profile in response to CYP-induced injury and MSC therapy in Sprague-Dawley female rats. A, Anti-Müllerian hormone (AMH), B, Estradiol (E2); C, Follicular stimulating hormone (FSH) and D, Luteinizing hormone (LH). CYP caused a decline in both AMH and E2, after one and two weeks which improved significantly ($p<0.0001$ and $p<0.001$, respectively) after MSC therapy, approaching those seen in the NC group. In contrast, CYP induces higher levels of FSH and LH than the NC group, where MSCs therapy resulted in significant ($p<0.0001$) improvements comparable to the NC group.All the data are presented as means ± SEM. *p<0.01; **p<0.001; ***p<0.0001. The comparison was done against the CYP group

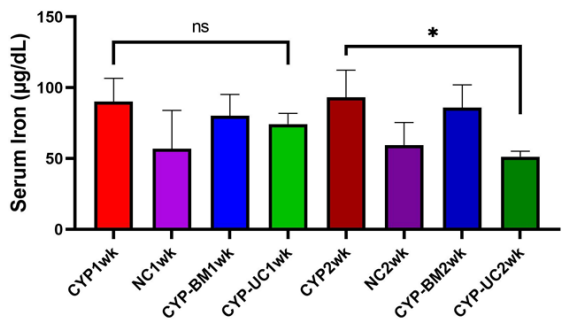

Figure 3: Serum iron levels change in response to CYP-induced injury and MSC therapy in Sprague-Dawley female rats. After two weeks of UC-MSC therapy, the CYP-induced serum iron overload level improved statistically significantly, approaching that of the NC group. All the data are presented as means ± SEM. *p<0.01. The comparison was done against the CYP group

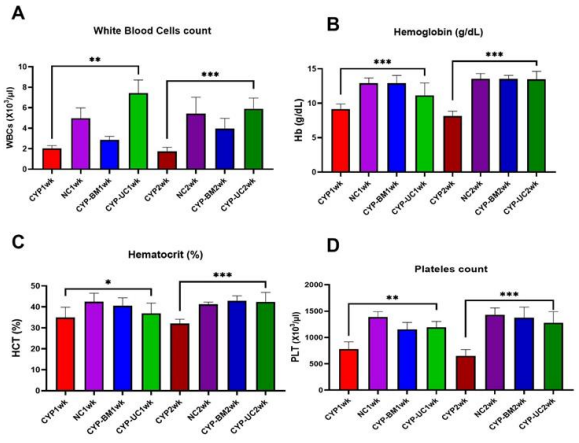

Figure 4: Hematological parameters change in response to CYP-induced injury and MSC therapy in Sprague-Dawley female rats. A, White blood cells (WBCs); B, Hemoglobin (Hb); C, Hematocrit (HCT) and D, Platelet (PLT). The CYP group had a considerably lower leukocyte count (P<0.0001), Hb concentration (P<0.0001), HCT% (P<0.0001), and thrombocyte count (P<0.0001), MSCs therapy ameliorated these decreases, UC-MSCs group responded better than BM-MSCs group (particularly in leukocyte count; P=0.001) after one week. This is because Hb, HCT%, and thrombocyte count did not vary. All the data is presented as means ± SEM. *p<0.01; **p<0.001; ***p<0.0001. The comparison was done against the CYP group

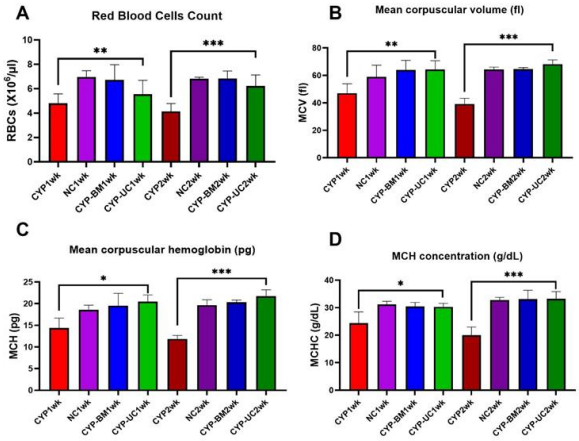

Figure 5: Hematological parameters change in response to CYP-induced injury and MSC therapy in Sprague-Dawley female rats. A. Red blood cells (RBCs); B, Mean corpuscular volume (MCV); C, Mean corpuscular hemoglobin (MCH), D, MCH concentration (MCHC). The CYP group had a considerably lower erythrocyte count (P<0.0001), and packed cell volume (PCV), and consequently MCV, MCH, and MCHC (P<0.0001) when compared to the NC group. MSCs therapy significantly restores these drops. All the data is presented as means ± SEM. *p<0.01; **p<0.001; ***p<0.0001. the comparison was done against the CYP group

4. DISCUSSION

Chemotherapeutic drugs generally destroy dividing cells in the body, including cancer cells and normal bone marrow cells. Phosphoramide mustard, CYP's active metabolite, produces DNA crosslinks, resulting in DNA strand breaks and, ultimately, chromosomal breakdowns (Voelcker, 2020; Xie et al., 2024). This research compares the protective effects of UC-MSCs and BM-MSCs against CYP-induced hormonal and hematological disturbances in female rats. The POF is a reproductive condition that causes infertility and has been a long-standing problem for women worldwide affecting the ovary in women under the age of 40. Recently, various types of stem cells, especially MSCs, have seen increasing use in reproductive medicine due to their qualities, such as immunoregulation, anti-inflammation, angiogenesis, anti-apoptosis, and trophicity. However, cell therapy's widespread clinical use is hampered by its safety, cost, and manufacturing (Gupta et al., 2022; Guo et al., 2023). The present study affirms that using MSCs therapy can improve the hormonal and hematological damage induced by CYP in female rats. The MSCs treatment has intriguing clinical applications, with BM-MSCs being the most extensively investigated. Interestingly, significant drawbacks to using BM-MSCs, first, the method of collecting BM-MSCs will cause suffering to patients. The effectiveness of BM-MSC transplantation is not as high as expected in clinical settings because the number of cells, and their differentiation and proliferation capabilities, decreases as patient's age. In contrast, UC-MSCs are cells derived from umbilical cord tissue with a high capacity for proliferation and differentiation. UC-MSCs have lower immunological competency than BM-MSCs, limiting their ability to elicit a systemic immune response and graft-versus-host disease. As a result, UC-MSCs may be an appropriate alternative for BM-MSCs in clinical application (Zhang et al., 2021; Huang et al., 2022; Umer et al., 2023). In the presented results UC-MSCs responded better than BM-MSCs, particularly after two weeks of therapy. In the present investigation, administering CYP 200mg/kg for two weeks caused a serious disruption in reproductive hormonal function in the CYP group as compared to the NC group. A considerable drop in AMH and E2 was seen, whereas FSH and LH levels increased significantly in the CYP group. Transplanting MSCs by tail vein injection heals the condition by modulating serum hormone expression and restoring ovarian function. These results proved that MSCs transplantation can exert beneficial effects on the POF model of rats. BMSC and UCMSC transplantation improved ovarian function, as reported by previous research (Wang et al., 2021; Zhang et al., 2021). Several studies have shown the beneficial effects of bone marrow stromal cell treatment in a chemotherapy-induced ovarian failure animal model. Specifically, the study indicated that bone marrow stromal cells restored ovarian structure and function (Mohamed et al., 2018). Although chemotherapy drugs can inhibit the growth of tumor cells, they can also lead to granulosa cell apoptosis, follicular atresia, ovarian function decline, and other manifestations of premature ovarian failure. Granulosa cells are located on the lateral side of the oocyte zona pellucidum and secrete estrogen under the action of follicle-stimulating hormone and other paracrine factors, which play a role in the nutrition and support of oocytes. Granulosa cell apoptosis thus decreases estrogen levels in the body, affecting the normal development of oocytes. Abd-Allah et al., (2013) used bone marrow stromal cells from male rabbits to treat cyclophosphamide-induced ovarian failure. The study found that the ovarian functional reserve and number of follicles were improved. In addition, there were increased estrogen and vascular endothelial growth factor levels, reduced follicle-stimulating hormone levels, and diminished caspase-3 expression. Badawy et al., (2017) showed that bone marrow stromal cells can restore ovaries damaged by chemotherapy in mice. Iron is a vital element for existence, and because of its electron exchange properties, it participates in oxygen transport, energy generation, DNA, RNA, and protein synthesis. No scientific evidence exists to confirm the relationship between elevated iron levels and chemotherapy, even though this observation has been made (Ochiai et al., 2013) It has been reported that serum iron levels may increase after medication with various anticancer drugs. The study explored the role of serum iron in the adverse effects of cancer chemotherapy (Miya et al.,2018; Piskin et al., 2022). In the present investigation, administration of the CYP 200 mg/kg for two weeks resulted in an iron overload level that was statistically significantly improved after UC-MSCs therapy, approaching that of the NC group, particularly after two weeks of UC-MSC treatment. The iron levels in the body do not show any

significant positive correlations with serum reproductive hormone levels. Properly, this study's findings expand current knowledge on the factors affecting iron stores and the endocrine system through the harmful deposition of iron in endocrine glands and hormonal effects on iron absorption and metabolism (Kadhum et al., 2021; Ni et al., 2022; Holzer et al., 2023). In the present research after administration of the CYP 200 mg/kg for 2 weeks, the CYP group showed significant hematological abnormalities compared to the NC group. The Hb concentration, leukocyte count, erythrocyte count, thrombocyte count, and mean volume of PCV, MCV, MCH, and MCHC all decreased significantly in the CYP group, where similar findings were reported (Kausar and More, 2019; Khazaei et al., 2020; Gözüoğlu and Yıldız, 2021; Şahin, 2022; Rajab et al., 2022). A prior investigation found that immunomodulatory agents are often used to reduce myelosuppression and enhance the immune response for cancer treatment. Cyclophosphamide (CYP) can induce oxidative stress in the bone marrow, resulting in the suppression of antioxidative enzymes and causing myelosuppression. Who added that, CYP-induced myelosuppression/ immunotoxicity restricted the usage of the CYP (Lee and Lim, 2013). In the present study, MSCs-administered groups in combination with CYP demonstrated a statistically significant increase in different hematologic cell levels particularly in the UC-MSCs treated group, where the levels drew near the control value ($p<0.001$). Previous investigations of treatments other than MSCs have revealed similar findings (Gözüoğlu & Yıldız, 2021; Şahin, 2022; Rajab et al., 2022).

5. CONCLUSIONS

In conclusion, the existing results showed that injecting CYP can hurt ovarian function and disturb the release of reproductive hormones. BM-MSCs or UC-MSCs therapy may be a promising alternative for patients with premature ovarian failure (POF), especially for those who suffer from chemotherapy-induced POF. This provides evidence for MSCs transplantation as useful strategy in future clinical trials. So, this work serves as the basis for the injection of BM-MSCs or UC-MSCs to facilitate the restoration of premature ovarian failure by promoting hormone secretion.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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