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Bone marrow derived mesenchymal stem cells transplantation rescues premature ovarian insufficiency induced by chemotherapy

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ABSTRACT

Premature ovarian insufficiency (POI) is an important cause of infertility and also cause menopausal symptoms, which greatly reduced the quality of life for women. Hormone replacement therapy (HRT), as an important strategy, improved the quality of life for patients, however, the role of HRT in promoting fertility remains controversial. Therefore, seeking an optimal regime for POI becomes more urgent. In this study, we established POI model induced by CTX and BUS and utilized bone marrow derived mesenchymal stem cells (BM-MSCs) transplantation to treat the POI. We found that the decrease of estrogen and the increase of FSH induced by administration of CTX and BUS were rescued by BM-MSC transplantation. H&E staining and TUNEL assay showed that there were more healthy ovarian follicles and less apoptosis of ovarian cells after treatment with BM-MSCs. Further studies showed that there was an obvious decrease of Bax, p53, and p21 after transplantation, however, CyclinD2 was increased. In conclusion, our results demonstrated that BM-MSCs could restore injured ovarian function. Inhibiting apoptosis and promoting residual ovarian cell proliferation may contribute to the process.

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POI; BM-MSC; transplantation; apoptosis; proliferation

Introduction

Premature ovarian insufficiency (POI), also known as primary ovarian failure, affects 1–2% of women under the age of 40. It is characterized by amenorrhea, hypoeurogenism, and hypergonadotropinism. The estradiol level is below 50 pmol/L and follicle stimulating hormone (FSH) level is greater than 40 mIU/mL [1,2]. Vaginal dryness, hot flushes, infertility, osteoporosis, and cardiovascular disease are the most common subsequent consequences, which largely diminish the quality of life in patients [3,4]. However, the etiology of POI remains sophisticated. Studies showed that X-chromosome abnormalities, autosomal genetic abnormalities, autoimmune disorder, and enzymatic defects were involved in the development of the disease [5–8]. The POI induced by chemotherapy drugs become more and more prominent [9,10]. A wide variety of hormone replacement regimes are employed to treat the POI, however, the role of hormone replacement therapy (HRT) in promoting fertility remains controversial. Compared with the normal population of women of reproductive age, the uterine volume and endometrial thickness are lower in POI patients who undergone HRT [11,12]. Thus, for patients with reproductive needs, seeking an optimal regime becomes an urgent task.

In the last two decades, stem cells therapy has become a promising method for various diseases, especially the mesenchyme stem cell (MSC) transplantation. MSCs refer to adult and fibroblast like multipotent cells. The cells can be easily isolated and expanded from various tissues including bone marrow, adipose tissue, umbilical cord blood, skin, muscle, lung, tendon, etc. [13]. Animal models and clinical trials proved the effectiveness of

MSCs transplantation in acute myocardial infraction, lung injury, septic shock, graft versus host disease, and liver cirrhosis [14–18]. MSCs exert their therapeutic effects mainly through homing to injured sites and secreting paracrine factors, such as vascular endothelial growth factor (VEGF), insulin growth like factor-1 (IGF-1), hepatocyte growth factor (HGF), and basic fibroblast growth factor (b-FGF) [19,20]. Encouraging studies have also demonstrated that umbilical cord blood derived mesenchymal stem cells, skin derived mesenchymal stem cells, human amniotic fluid stem cells, and human amniotic epithelial cells can rescue chemotherapy-induced ovarian damage [21–24]. Nevertheless, detailed mechanisms remain to be elucidated.

In this study, we established POI models through intraperitoneal injection of cyclophosphamide and busulfan, as described by Wang et al. [25]. We observed the influence of BM-MSCs transplantation on follicle development and hormone secretion induced by chemotherapy and explored the possible mechanism.

Materials and methods

Experimental animals

All animal procedures were conducted in accordance with the guidelines of ethical committee of Nanchang University for animal care and use. Female Kunming mice were purchased from Animal Facility of Nanchang University and housed under a temperature and light controlled conditions with free access to water and food, which allowed the mice to acclimatize in the

laboratory conditions for one week prior to experimental procedures.

Animal model establishment

The 24 mice were randomly divided into three groups, including control group (con group), chemotherapy-induced POI group (POI group) and MSC transplantation group (POI+MSC group). To establish the POI model, the mice received a single intraperitoneal injection of cyclophosphamide (120 mg/kg), and immediately followed by subcutaneous injection of busulfan (30 mg/kg). The mice in the control group received the same volume of 0.9% saline. Mice in the POI+MSC group received MSC transplantation at 7 days post-induction. One month later, ovaries were dissected from mice for further studies. 1 mL whole blood was also collected and serum was stored at -80°C for estrogen and FSH examination.

Isolation and culture of MSCs

The tibiae and femurs of female Kunming mice were removed and bone marrow plugs were flushed with phosphate-buffered saline (PBS) solution. The cell suspension was layered after mixed with Percoll solution and centrifuged at 2500 r/min for 20 min. Cells at interface were harvested and washed twice with PBS. The cells were cultured with DMEM/F12 culture medium, supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 mg/mL streptomycin sulfate and at 37°C in a humidified atmosphere of 5% CO_2 . Three days later, the non-adherent cells were removed according to the unique plastic-adherent property of MSC as documented by Poliseti et al., which provide the double guarantee of the BM-MSCs purity [26]. When the density of cells reached 80–90% confluency, the cells were passaged. The MSCs transplantation was performed using the second passage.

Stem cell transplantation

One week after the establishment of the POI mouse model, the mice received MSCs transplantation by tail intravenously. MSCs were resuspended in PBS at 4°C for less than 30 min before transplantation. Each POI mouse was transplanted with $100\ \mu\text{L}$ MSCs ($1 \times 10^6/\text{mL}$) via a microinjector.

Estrogen and FSH measurement

The serum samples were obtained from three different groups of mice and stored at -80°C for subsequent hormone measurement. The estrogen and FSH were assessed by a commercial laboratory (Beijing Sino-uk Institute of Biological Technology, Beijing, China) using the commercial radioimmunoassay kit.

Hematoxylin and eosin staining

Ovaries were immediately fixed in 4% paraformaldehyde at 4°C for 24 h and stored in 70% ethanol. The samples were then embedded in paraffin blocks. $5\ \mu\text{m}$ sections were cut. After deparaffinization and rehydration through gradient ethanol elution, hematoxylin and eosin (H&E) staining and mounting were carried out.

TUNEL assay

Terminal dextrynucleotidyl transferase (TDT)-mediated dUTP nick end labeling (TUNEL) assay was performed using the Dead End

Apoptosis Detection Kit (Promega, Madison, WI) according to the manufactures' instruction. Briefly, the paraffin sections were dewaxed and rehydrated. Tissues were then fixed with 4% PFA for 15 min and treated with proteinase K at room temperature for 10 min. The slides were washed twice with PBS and incubated with a reaction mix containing Nucleotide mix (including fluorescein-12-dUTP) and TDT for 1 h at 37°C . After washed with 2XSSC and PBS, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Apoptotic cells with Green fluorescence were examined by OLYMPUS fluorescence microscope.

RNA extraction and real-time PCR

Total RNA was extracted from ovaries using Trizol Reagent (Invitrogen, Carlsbad, CA), and the quality of the RNA was determined using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). $2\ \mu\text{g}$ total RNA was reversely transcribed into cDNA. The sequences for specific primers are listed in Table 1. Real-time PCR was carried out in a $20\ \mu\text{L}$ reaction volume, which consisted of $10\ \mu\text{L}$ 2XBrilliant SYBR Green QPCR Master Mix (TIANGEN, Beijing, China), $1\ \mu\text{L}$ cDNA, $0.5\ \mu\text{L}$ primers, and $400\ \text{nM}$ of passive reference dye using ABI thermal cycler 7500. The reaction was under the following conditions: 94°C for 30 s, 40 cycles of 94°C for 5 s, and 60°C for 30 s. Melting curve analysis was conducted to analyze the purity of products after the real-time PCR. Experiments were repeated three times and each gene was set a parallel hole.

Statistical analysis

Graph Pad Prism v6.01 (Graph Pad Software Inc., San Diego, CA) was used to analyze the data. Data are presented as the mean and standard deviation (SD). One-way ANOVA followed by a least-significant-difference test was used for statistical comparisons among multiple groups. A p values less than .05 was deemed as statistically significant.

Results

BM-MSCs transplantation relieved chemotherapy-induced follicular dysplasia and abnormal hormone secretion

Bone marrow derived MSCs were adherent and morphologically resembled fibroblasts. Cells of the second passage of MSC are shown in Figure 1(A). After MSCs transplantation, we examined the histological change of ovaries and serum hormone level. We

Table 1. The oligonucleotides used for real-time PCR.

| Gene | Sense and antisense primer |
|----------|--|
| GAPDH | 5'TCCTTGGAGGCCATGTAGGCCAT3 5'TGATGACATCAAGAAGTGGTGAAG3' |
| CyclinD2 | 5'GCCAAGATCACCCACT3' 5'-GCTGCTCTGACGGAACT3' |
| Bcl2 | 5'CTCGTCTACCGTCTGACTTCG3' 5'CAGATGCCGTTTCCAGTACTCAGTC3' |
| Bax | 5'ACCAGCTCTGAACAGATCATG3' 5'TGGTCTGGATCCAGACAAG3' |
| Caspase3 | 5'AGGGGTCATTTATGGGACA3' 5'-TACACGGGATCTGTTTCTTTG3' |
| P21 | 5'-TTGTCGCTGCTTGCCTCT3' 5'-AATCTGTCAGGCTGGTCTGC3' |
| P27 | 5'-GCAGGAGAGCCAGGATGTCA3' 5'-CCTGGACTGCTCCGCTAA3' |
| P53 | 5'-GTTCCGAGAGCTGAATGAGG3' 5'-TTATGGCGGAGGTAGACTG3' |

found that there were a typical size and distinct stages of follicles, ranging from primordial follicle to corpus luteum in the control group (Figure 2(A)). After CTX and BUS administration, there was an abatement of ovarian follicle number (Figure 2(B)). After BM-MSCs transplantation, large number of healthy follicles were shown in ovaries (Figure 2(C)). The serum estrogen and FSH level are shown in Figure 1(B,C). After administration of CTX and BUS, the mean serum estrogen level was significantly decreased compared to the control group. It was rescued by BM-MSCs transplantation. FSH expression level was increased greatly in the POI group compared with the control group. After MSCs transplantation, the FSH level was also decreased.

BM-MSCs transplantation rescued chemotherapy-induced ovarian cell apoptosis

Previous studies showed that chemotherapy-induced ovarian cell apoptosis and stem cell can reduced ovarian cell apoptosis [23,27]. We also examined it through TUNEL apoptosis assay. As shown in Figure 3(C-F), TUNEL-positive cells were observed in the ovarian tissue section after CTX and BUS induction. Compared with the control group (Figure 3(A,B)) the apoptosis of granulosa cells and theca cells in POF mice were increased. However, this phenomenon was relieved in the BM-MSCs transplantation group (Figure 3(G-J)). We also examined the expression of apoptosis related genes, including P53, Bax, Bcl2, and caspase3 through real time-PCR. As shown in Figure 4(A), p53 was elevated after chemotherapy induction, and returned to normal level after receiving BM-MSCs transplantation. Bax, a pro-apoptotic gene, was also increased in the POF group and was inhibited by BM-MSCs (Figure 4(B)). Bcl2 and caspase3

expression level were decreased in POI, whereas, no restoration was found after BM-MSCs transplantation (Figure 4(C,D)).

BM-MSCs transplantation promoted the proliferation of residual ovarian cells via regulating CyclinD2 and p21 expression

After BM-MSCs transplantation, we found that the follicles grew healthily. The growth of follicles was characterized by ovarian cell proliferation. So, we inferred that the proliferation was also influenced by BM-MSCs. We examined the expression of proliferation related genes, including CyclinD2, p21, and p27. As shown in Figure 5(A), CyclinD2 was dramatically elevated and p21 was greatly decreased after BM-MSCs administration (Figure 5(C)). The expression p27 was not changed among these groups (Figure 5(B)). These results suggested that BM-MSCs could not only inhibit apoptosis induced by chemotherapy, but also play a vital role in promoting residual ovarian cells proliferation.

Discussion

Alkylating agents, CTX and BUS, were widely used to improve the long-term survival of cancer patients [28]. However, the serious impairment on ovaries, such as POI, became increasingly concerned [29]. Our results showed that administration of CTX and BUS diminished ovarian follicle development and disturbed endocrine hormone (estrogen and FSH) secretion.

Stem cells transplantation is a promising therapeutic tool in rehabilitating ovarian function and fertility [30]. Diversified types of mesenchymal stem cells, including umbilical cord-derived

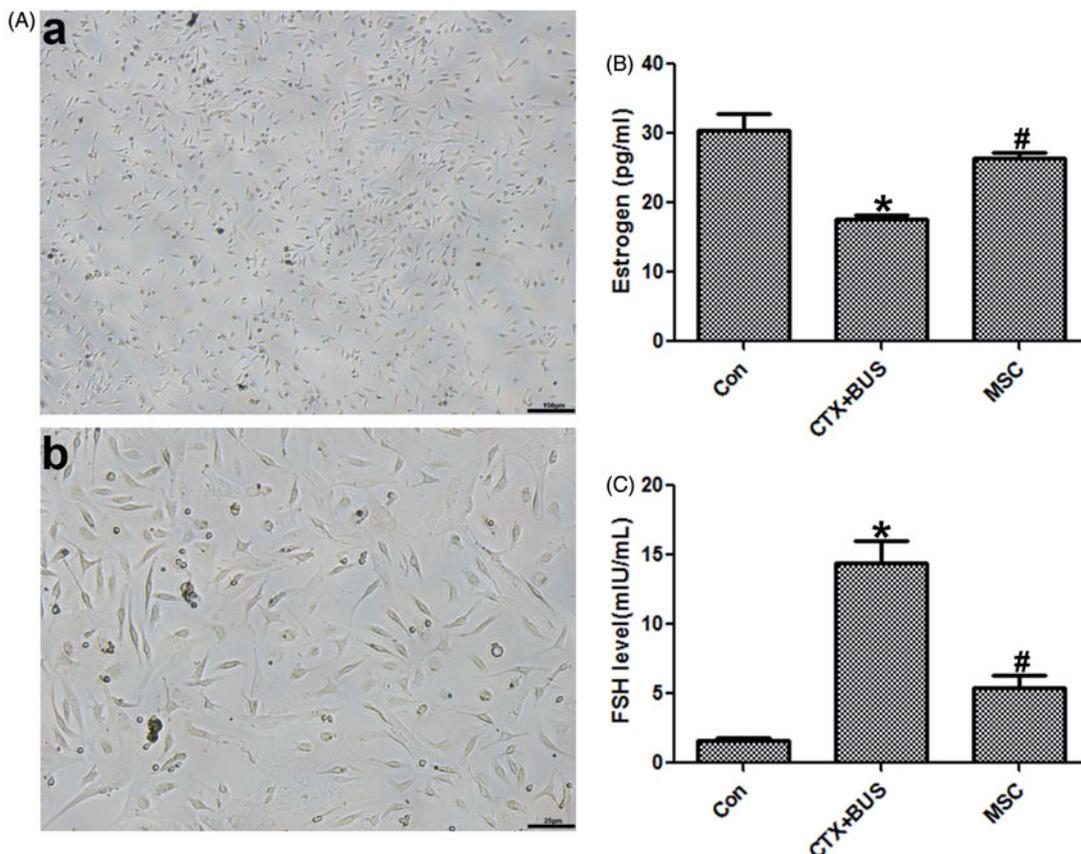


Figure 1. (A) The typical fibroblastic morphology was shown by second passage BM-MSCs. The estrogen (B) and FSH (C) expression level were assessed by radioimmunoassay. Bar graphs show mean \pm standard error of mean (SEM) from three independent experiments. * $p < .05$ vs. control group, # $p < .05$ vs. CTX + BUS group.

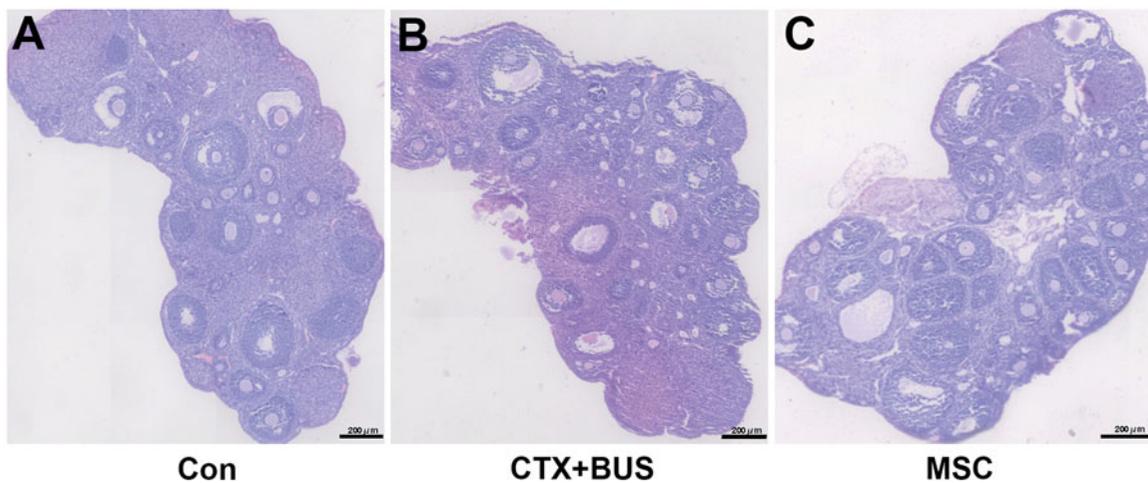


Figure 2. H&E staining of ovarian morphology in control (A), chemotherapy induced (B), and BM-MSCs transplanted (C) groups.

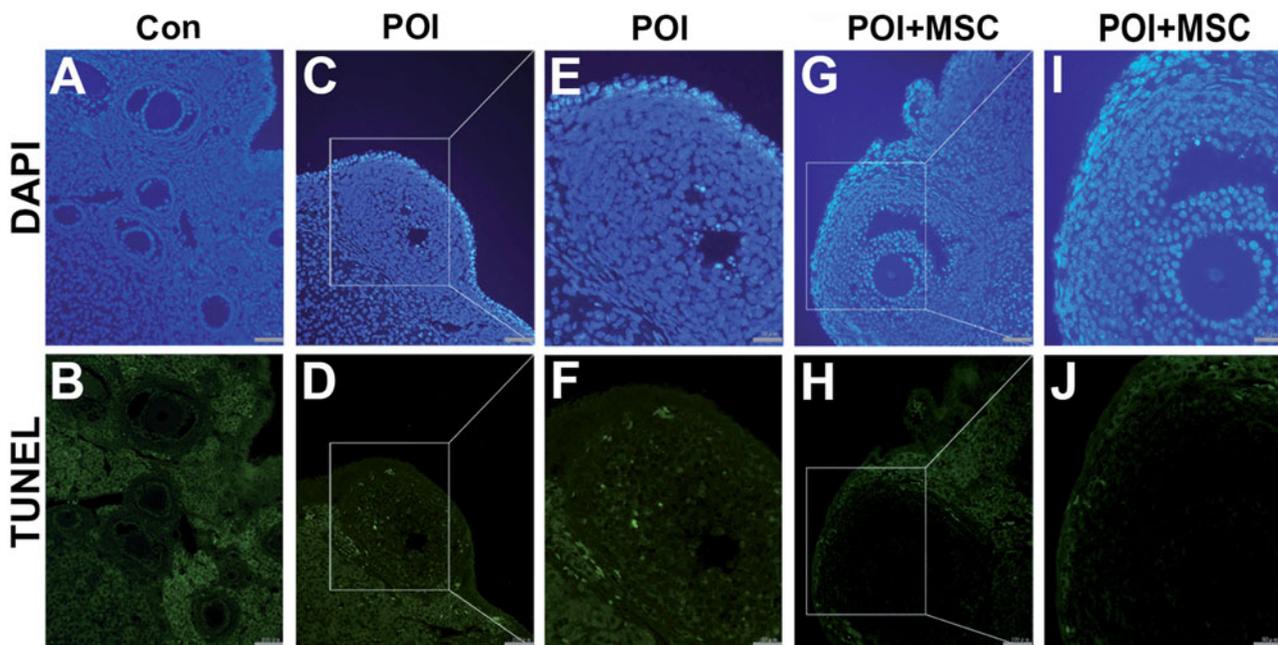


Figure 3. Cell apoptosis assay: chemotherapy-induced apoptosis of granulosa cells and the thecal cells within follicles of the POI group (C, D, E, and F). The control group (A and B) and BM-MSCs transplanted group (G, H, I, and J) have also been examined.

mesenchymal cells, adipose-derived mesenchymal cells, and skin-derived mesenchymal cells have been tested to treat POI [22,24,31]. Although lots of achievements have been made, but the exact mechanism of mesenchymal stem cell transplantation on POI remains unclear. In this study, we observed the effect of BM-MSC transplantation on chemotherapy-induced POI mice. We found that BM-MSC transplantation relieved chemotherapy-induced follicular dysplasia. FSH level and estrogen level were also rescued. These results suggested that BM-MSC transplantation indeed reversed the ovarian damage induced by chemotherapy. Apoptosis is a main outcome of chemotherapeutic treatment [32]. We inferred that chemotherapy-induced follicular dysplasia was related to the apoptosis of ovarian cells. We found that the apoptosis of ovarian cells were increased after administration of chemotherapy drugs. BM-MSCs transplantation rescued chemotherapy-induced ovarian cell apoptosis, which was consistent with previous studies [19,33]. We also examined the expression

of apoptosis related genes, including Bax, p53, caspase3, and Bcl2. Transcription factor p53 was demonstrated to be an important apoptosis inducer in mammalian ovarian cells [34]. During follicle maturation and atresia, p53 promotes granulosa cells apoptosis and basal progesterone secretion [35]. Bax, one downstream molecule of p53, could interact with mitochondrial voltage-dependent anion channel (VDAC) and lead to alteration of membrane potential and release of cytochrome c [36]. The role of Bax in promoting apoptosis of granulosa cells during follicle development have been proved [37,38]. We found that p53 and Bax expression was increased after chemotherapy induction and BM-MSCs transplantation downregulated the expression of p53 and Bax. These data suggested that apoptosis inhibition contributed to the effect of BM-MSCs on resumption of ovarian function.

In fact, the growth of normal follicles is characterized by ovarian cell proliferation, especially granulosa cells. These ovarian

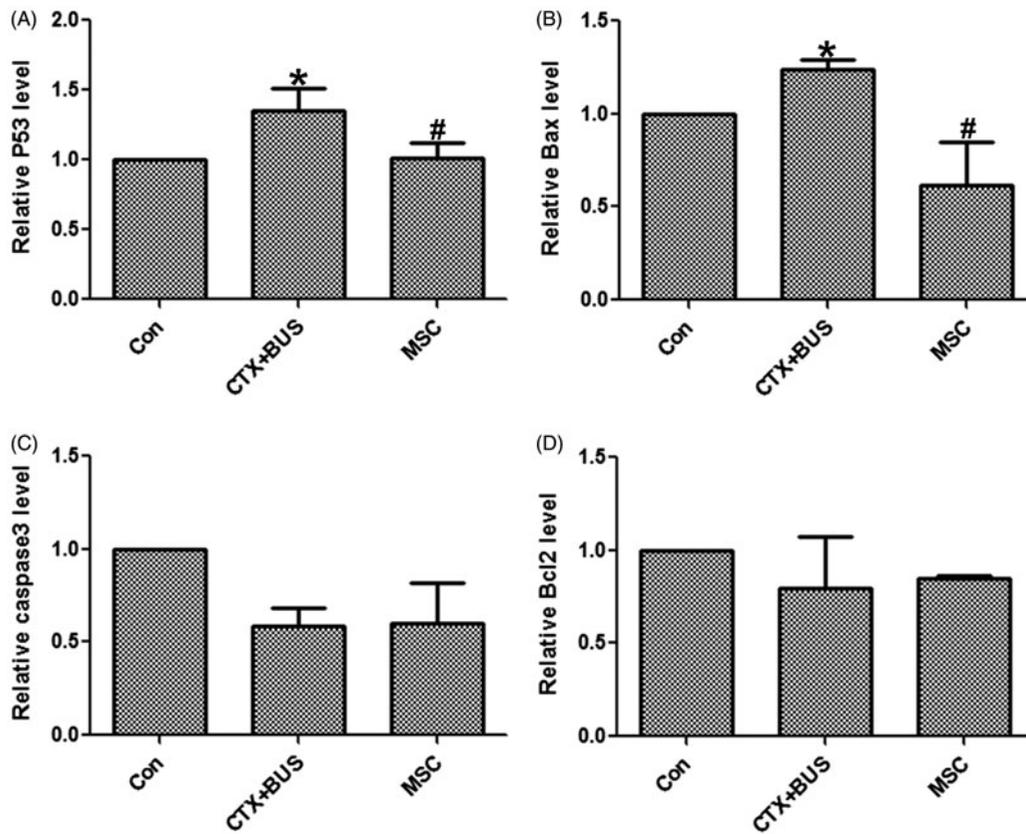


Figure 4. Alteration of p53 (A), Bax (B), caspase3 (C), and Bcl2 (D) mRNA expression level in ovarian tissue was examined by real time-PCR. Mean \pm standard error of mean (SEM) from three biological replicates. * $p < .05$ vs. control group, # $p < .05$ vs. CTX + BUS group.

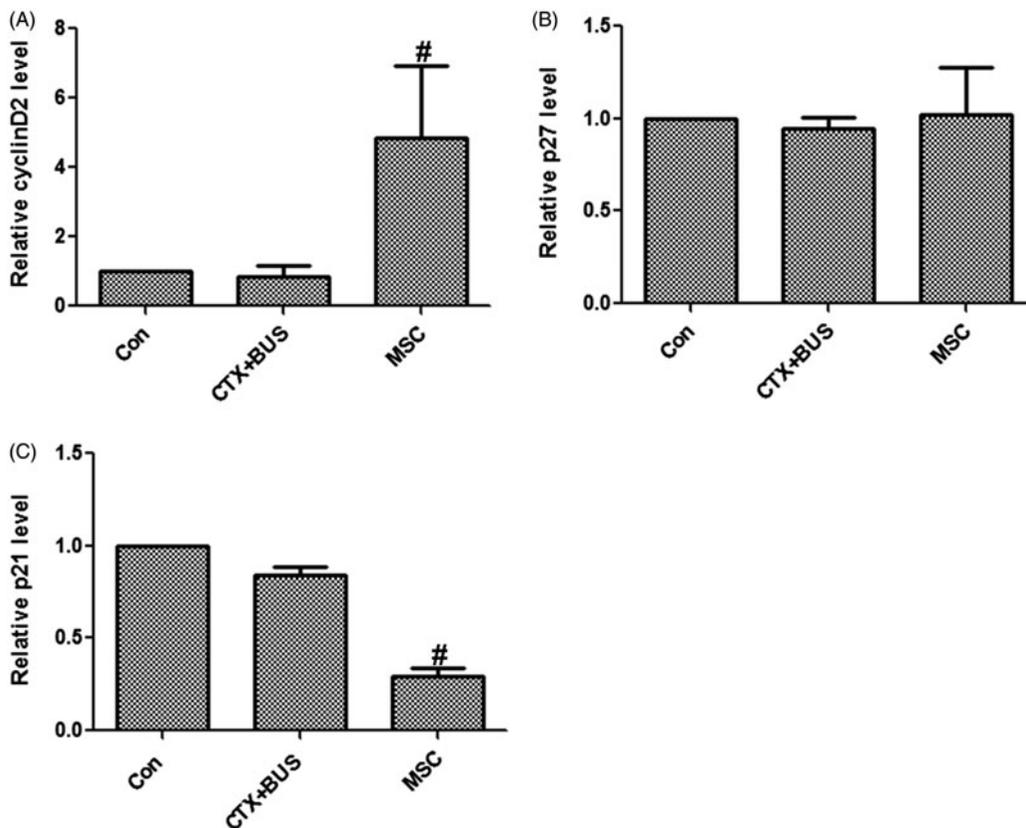


Figure 5. Relative expression of proliferation markers including CyclinD2 (A), p27 (B), and p21 (C) expression level in ovarian tissue was detected among different experiment groups by real time-PCR. Values represent the mean \pm standard error of mean (SEM) from three independent experiments. * $p < .05$ vs. control group, # $p < .05$ vs. CTX + BUS group.

cells work together to synthesize estrogen and regulate normal reproductive function [39]. Overapoptosis of ovarian cells induced by chemotherapy drugs caused a declined serum estrogen level. However, the recovery of estrogen level should be associated with the increase of ovarian cell proliferation. To date, almost all studies focused on the anti-apoptosis function and underneath mechanism of MSCs in POF treatment. Encouraging results from our study revealed that BM-MSCs increased the CyclinD2 expression and decreased p21 expression. CyclinD2 is critical in promoting cell cycle progress. CyclinD2 deficiency in ovarian granulosa cells influenced the proliferation and caused infertile of females [40]. p21, known as cyclin-dependent kinase inhibitor 1A (CDKN1A), exerts its function through binding to cyclins and cyclin-dependent kinases (CDKs) directly, which lead cell arrest at G1/S and G2/M transitions [41]. Cell cycle progression is precisely regulated by cyclins and several CDKs. Based on our results, we inferred that BM-MSCs transplantation regulated CyclinD2 and p21 expression and increased residual ovarian cell proliferation and restored ovarian function.

Several studies indicated that expression of CXC chemokine receptor 4 (CXCR4), platelet-derived growth factor receptor (PDGFR)- β , $\alpha 5\beta 1$ -integrin, and junction adhesion molecule A (JAM-A) on MSC confer the homing ability to injured sites [42–45]. MSCs exert their therapeutic effects mainly through homing to injured sites and secreting paracrine factors, such as VEGF, IGF-1, HGF, and b-FGF [19,20]. VEGF and b-FGF cytokines can inhibit the apoptosis of granulosa cells and IGF-1 can promote the ovarian cells proliferation [46–48]. So we speculated that the anti-apoptosis and promoting proliferation activity of BM-MSCs were related to these cytokines.

In addition, studies showed that oogonial stem cells (OSCs) existed in human ovaries [49]. Further researches showed that these rare cells can stably propagate for long term and form follicles surrounded by granulosa cells rapidly *in vivo* [50]. The OSCs bring great promise for POI, however, more studies are needed to substantiate the possibility.

In conclusion, we demonstrated that transplanted BM-MSCs could rescue the chemotherapy-induced POI through inhibiting apoptosis and promoting residual ovarian cells proliferation. Apoptosis and proliferation genes, including p53, Bax, CyclinD2, and p21 may contribute to it. This possible mechanism of transplanted BM-MSCs will shed light on POI patients treatment. Nevertheless, more accurate molecular mechanism underneath BM-MSCs transplantation in POF and the safety of transplantation still need to be investigated in future work.

Disclosure statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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