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RESEARCH ARTICLE

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The effect of bone marrow-derived mesenchymal stem cells on chemotherapy induced ovarian failure in albino rats

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Abstract

Objectives: Chemotherapy targets rapidly dividing tissues in the body. It destroys the progenitor cells in gonads resulting in premature ovarian failure. Studies have suggested that bone marrow-derived stem cells can generate oocytes in chemotherapy treated female rats after transplantation. The present study aimed to assess mechanism of homing, the action of injected BM-MSCs on ovarian function after ovarian damage. **Experimental design**: Seventy two female albino rats were randomly allocated into Control and CTX group, The Experimental protocol was lasted for 12 weeks during which serum FSH and E2 were monitored twice at the end of the 2nd week (12 rats) and 8th week (6 rats). Stem cells identification and homing were evaluated by Flowcytometry and tagging of stem cells with iron oxide particles respectively. Also, histopathological examination was done to evaluate both degeneration (6 rats at 4th week) and regeneration (6 rats at 12th week) of ovarian tissue together with assessment of the levels of TNF-α in ovarian homogenate and IGF-I as a growth factor in ovarian tissue. **Principal observations**: Partial improvement of E2 and FSH levels as well as ovarian architecture. Elevation of ovarian TNF- α levels and of IGF-I immunohistochemical expressions in ovarian tissues of BM-MSCs injected rats were noticed following homing of BM- MSCs in the ovarian stroma in both control and chemotherapy groups. **Conclusion**: Injected BM- MSCs can home in the stroma of the injured ovaries. IGF-I and TNF- α may have a role in the attraction of stem cells in vivo.

KEYWORDS
bone marrow, MSCs, cyclophosphamide, ovary, Egypt

1 | INTRODUCTION

Cyclophosphamide is the most commonly implicated agent in causing damage to oocytes and granulosa cells in a dose-dependent manner (Kenney et al., 2001). The number of surviving primordial follicles following exposure to chemotherapy is in reverse correlation with the dose of chemotherapy (Meirow et al., 1999*)*. The follicular destruction generally results in loss of both endocrine and reproductive function, depending on the dose and the age of the patient (Larsen et al., 2003).

Mesenchymal stem cells (MSCs) have an inherent ability for self-renewal, proliferation, and differentiation toward mature tissues, depending on the surrounding microenvironment. Such characteristics intrinsic to stem cells make MSCs very attractive for use in cell therapy and regenerative medicine (Ra et al., 2011).

An important role in MSC-mediated protection is its inhibition of tissue apoptosis and augmentation of tissue turnover (Zhang et al., 2011). This is done through increasing prosurvival factors as Prokinase B (PKB/Akt) expression in injured cells (Morigi et al., 2004), growth factors as Insulin Growth Factor (IGF), Vascular Endothelial Growth Factor (VEGF) and Hepatocyte Growth Factor (HGF) expression which inhibit apoptosis and stimulate cell proliferation (Imberti et al., 2007; Lu et al., 2011). Also, some studies have suggested that MSCs possess anti-oxidative characteristics, as MSCs have been observed to produce many anti oxidative mediators such as IGF, Platelet Derived Growth Factor (PDGF), superoxide dismutase (SOD), HGF and Interleukin −6 (IL-6) (Hui et al., 2010).

TABLE 1 Subgroups and injection schedules of cases

	Control Cases	Chemptherapy (CTX) Cases
Subgroup A (12 rats)	with no injection	cyclophosphamide injection for 15 days
Subgroup B1 (Saline Injection) (12 rats)	intraperitoneal (Ip) saline injection for 15 consecutive days (Injection-1), followed by one intravenous (Iv) saline injection at (injection-2)	cyclophosphamide injection for 15 consecutive days, followed by one Iv saline injection in (injection-2).
Subgroup B2 (Saline And MSCs) (12 rats)	Ip saline injection for 15 consecutive days (Injection-1), followed by Iv one injection of BM-MSCs (injection-2).	cyclophosphamide injection for 15 consecutive days, followed by receiving one Iv injection of BM-MSCs (injection-2).

2 | MATERIALS AND METHODS

The study was conducted on 90 female albino rats, and the experimental protocol lasted for about 12 weeks.

All animal work was carried out under protocols approved by the Laboratory Animal Care and Use Regulations of Kasr Al Aini, Cairo University.

2.1 | Experimental animals

Ninety (90) adult female albino rats within reproductive age (6 − 10 weeks) were used in this study and their weights ranged from 200 to 250 g. All rats were evaluated by vaginal smears and only rats exhibiting at least two consecutive 4–5 days estrous cycles were included.

All rats were kept in chip-bedded cages (27×38×17cm) in Kasr Al Aini hospitals animal house, Cairo University, at room temperature under normal (12/12) day night cycle and were given free access to chow and water for the entire duration of study. The protocol was approved by the research institutional ethics committee of the departments of Clinical Pathology and Medical physiology on animal research, as well as the animal house- Faculty of Medicime-Cairo University. The study followed the International Society of Applied Ethology (ISAE) guidelines concerning animal rights.

Eighteen rats were considered from the start as donors. This donor group was kept under the same laboratory conditions of the other groups and from which bone marrow was harvested from their tibiae and femurs to obtain MSCs.

The remaining 72 rats were divided equally into 2 main groups; control group and chemotherapy (CTX) group. These groups were further divided into 3 subgroups (as 12 rats in every subgroup).

2.2 | Duration of the study

This study lasted for 12 weeks, and it was divided into the following time periods and points.

2.2.1 | Conditioning period (Cp)

One week of conditioning in the animal house of Kasr El Aini, Cairo University

2.2.2 | Injection-1 period

This period lasted 15 days during which the animals were divided mainly into control and chemotherapy groups (CTX). The CTX groups received (cyclophosphamide) to induce ovarian failure. While the control groups received intraperitoneal (Ip) saline injection (group B1) or no injection (group A).

2.2.3 | Monitoring-1 period (M1)

This period lasted for 1 week during which the animals in all groups were monitored by serum FSH, E2 levels, and vaginal smears.

2.2.4 | First tissue sampling point (Sp-1)

Six animals in each subgroup were sacrificed for ovarian tissue histopathology evaluation and TNF-α level in ovarian homogenates.

2.2.5 | Injection-2 point

The rest of the animals (6Rats) in each group were injected intravenously (IV) once with either stem cells (subgroup B2), saline (subgroup B1), while (subgroup A (did not receive any injection (Table 1).

2.2.6 | Monitoring-2 period (M2)

Serum samples for FSH and E2 were monitored twice at 2 points of time in this period which lasted for 8 weeks, M2 (a), at the end the 2nd week and M2(b), at the end the8th week.

2.2.7 | Second tissue sampling point (Sp-2)

At the end of the 12th week, all animals were sacrificed and ovarian tissue samples were taken to evaluate ovarian histopathology and the ovarian levels of IGF-I by immunohistochemistry.

2.3 | Induction of ovarian failure (chemotherapy protocol)

Intraperitoneal injection of 50 mg/kg of cyclophosphamide (Endoxan®, Baxter, Germany), as a loading dose followed by daily Ip injection of 8 mg/kg cyclophosphamide for 14 consecutive days (Abbasy et al., 2010).

2.4 | Vaginal smear

Vaginal secretion was collected in the early morning by inserting a plastic pipette filled with 10 μl of normal saline (NaCl 0.9%) into the rat vagina, The appearance of cornified cells was used as an indicator of estrogenic activity (Mandl, 1951).

2.5 | Blood sample collection and animal sacrifice

Blood samples were withdrawn from retro-orbital venous plexus for FSH & E2 assay, then left to clot for 15 min and centrifuged at 3000

(rpm) for 20 min then separated serum was kept frozen at −70°C till analysis of FSH (Sun Red biotechnology Company, Shanghai; Catalogue No. 201-11-0183) and E2 (Sun Red biotechnology Company, Shanghai; Catalogue No. 201-11-0175) by enzyme-linked immunosorbent assay (ELISA).

The animals were sacrificed by cervical dislocation and dissected out, the ovary specimens were collected & prepared according to:

A) Histological Study: Hematoxylin and eosin (Kiernan, 2001).
B) Immunohistochemical Study for ovarian tissue IGF-I: by using Anti-IGF-I Polyclonal Antibody (Bioss, Inc. Company, USA); Catalogue No. bs-0014R
C) Homogenization of ovarian tissue and TNF-α testing:

Ovarian tissues were immersed into ice-cold homogenization medium (HM), were transferred into a 50-ml beaker (on ice) and minced with scissors, so that the pieces of tissue are no more than 30 mm^3. Minced tissue was suspended in 40 ml HM, stirred and then the liquid decanted. Process is repeated 3 times and finally tissue was suspended in 40 ml of HM, agitated and rapidly transferred to the glass vessel of the homogenizer. Tissue was filtered through nylon to remove undisrupted cells and connective tissue. The tissue homogenate was transferred into a glass jar with Teflon-lined lid, was stored at −10C with lid loosely attached to allow sublimation of CO_2 overnight, then tightened on the homogenate container and stored at −10C. TNF-α testing was done using ELISA kits (eBioscience Company, USA; Catalogue No. BMS622).

2.6 | Bone marrow-derived MSCS isolation, separation and expansion

By flushing, the excised tibiae and femurs of the donor group through a 20 gauge needle filled with culture medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum), bone marrow was harvested in heparinized conical tubes

Separation of mononuclear cells was done according to the method of, Waheed et al., 2010, under aseptic conditions for all cases: The harvested bone marrow cells were gently pipetted to break up cell clumps, the cells were centrifuged at 2000 rpm for 20 min, then, the cell pellet was resuspended carefully onto 5ml of 60% Ficoll Hypaque separating solution in sterile conical tube, and then centrifuged for 20–25 min at 2000 rpm at 8°C.

The mononuclear cells were retrieved from the buffy coat layer by sterile Pasteur pipette and placed in 5 ml sterile conical tube. The cells were washed two to three times with PBS, and centrifuged at 2000 rpm for 20 min. Cells were counted using automated cell counter (Cell Dyne, Inc, USA).

The cell suspension was seeded in three T25 tissue culture flasks, in a density of 100.000 cells in each flask, with 5 ml culture medium, at 37CÊ in a humidified atmosphere with 5% CO_2 for 2 weeks, Flasks

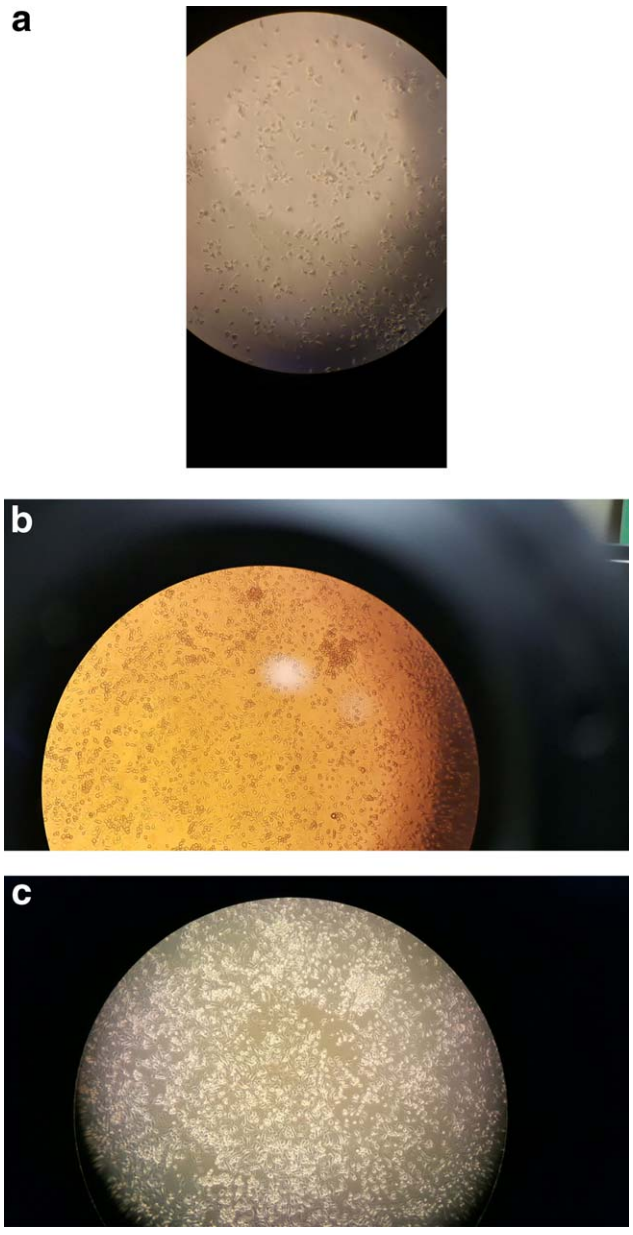

FIGURE 1 (a) BM-MSCs following isolation 300DPI. (b) BM-MSCs 3days following isolation 300DPI. (c) BM-MSCs of 80% confluence, fusiform-shaped 300DPI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were observed and refeed every 3 days, and passaged when the BM-MSCs have reached ~80% confluence. The mesenchymal population was isolated on the basis of its fusiform shape and its ability to adhere to the culture plate (Figure 1).

Harvest of BM-MSCs population was done using Trypsin 0.25 EDTA, and Trypan blue dye exclusion test was tried to assess the cell viability.

Flowcytometry analysis of cell surface molecules: CD29 (Biolegend, USA) and CD90 (eBioscience, USA), were detected onto FACS Caliber (Beckman Coulter, NE15106).

Cells were counted using cell counter (Cell Dyne Inc, USA), tagged with iron oxide particles; 100 μL Feridex (Feridex, Bayer Health Care)

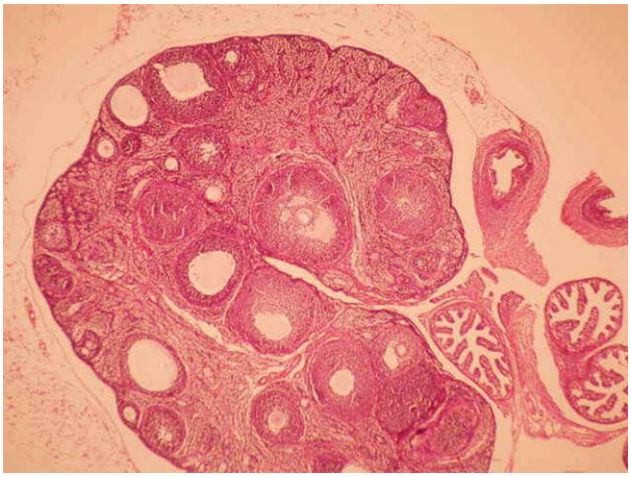

FIGURE 2 Normal ovarian tissue Group A control 300 DPI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

to 500.000 cells for 30 min, suspended in saline 0.9% NaCl; and injected into rats (1×10^6) MSCs in 0.25 ml saline intravenously for every rat.

Iron particles were visualized by Prussian blue staining, and examined under an inverted light microscope (Leitz, Germany) (Vallée et al., 2012).

2.6 | Detailed methodology protocol of different subgroups (Table 1): control group a (Figure 2)

Conditioning period for 1 week →no injection during (injection-1) period→ monitoring for FSH, E2 and vaginal smear during (monit-1) → sampling and sacrificing of 6 rats in (sp-1) for ovarian histopathology and level of TNF-α→ receiving no injection during (injection-2) stage→ 2nd monitoring for FSH, E2, and vaginal smear during (monit-2) → sacrificing to evaluate ovarian histopathology and the level of IGF-1 in (sp-2).

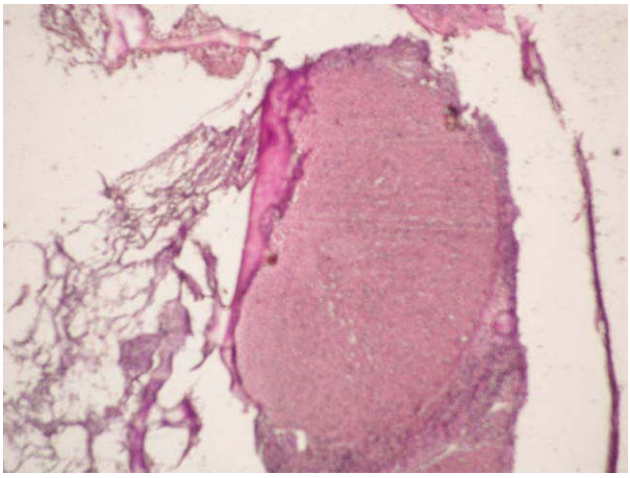

FIGURE 3 Ovarian tissue following CTX injection 300DPI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

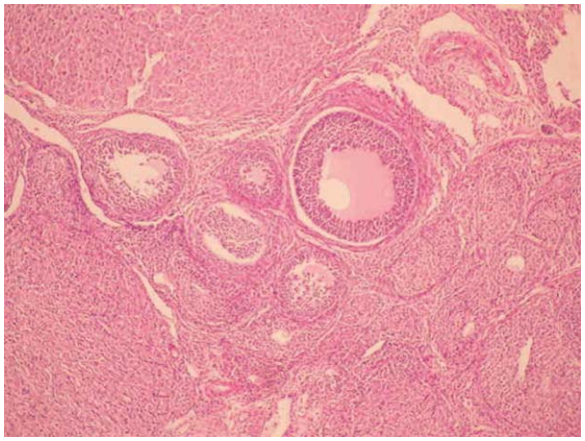

FIGURE 4 Ovarian tissue following BM-MSCs injection 300DPI. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

2.7 | Control group B1

Conditioning period for 1 week → injection of Ip saline for 15 consecutive days during (injection-1) period→ monitoring for FSH, E2, and vaginal smear during (monit-1) →sampling and sacrificing of 6 rats in (sp-1) for ovarian histopathology and level of TNF-α→r eceiving Iv saline injection once during (injection-2) stage→ 2nd monitoring for FSH, E2, and vaginal smear during (monit-2) →sacrificing to evaluate ovarian histopathology and the level of IGF-1 in (sp-2).

2.8 | Control group B2

Conditioning period for 1 week → injection ofIp saline for 15 consecutive days during (injection-1) period→ monitoring for FSH, E2, and vaginal smear during (monit-1) →sampling and sacrificing of 6 rats in (sp-1) for ovarian histopathology and level of TNF-α→ receiving Iv MSCs injection once during (injection-2) stage→ 2nd monitoring for FSH, E2&vaginal smear during (monit-2) →sacrificing to evaluate ovarian histopathology and the level of IGF-1 in (sp-2).

2.9 | CTX group a: (Figure 3)

Conditioning period for 1 week →induction of ovarian failure by cyclophosphamide during (injection-1) period→ monitoring for FSH, E2, and vaginal smear during (monit-1) →sampling and sacrificing of 6 rats in (sp-1) for ovarian histopathology and level of TNF-α→ receiving no injection once during (injection-2) stage→ 2nd monitoring for FSH, E2&vaginal smear during (monit-2) →sacrificing to evaluate ovarian histopathology and the level of IGF-1in(sp-2).

2.10 | CTX group B1

Conditioning period for 1 week →induction of ovarian failure by cyclophosphamide during (injection-1) period→ monitoring for FSH, E2 and vaginal smear during (monit-1) →sampling and sacrificing of 6 rats in (sp-1) for ovarian histopathology and level of TNF-α→ receiving IV saline injection once during (injection-2) stage→ 2nd monitoring for

TABLE 2 Serum levels of biomarkers, and number of ovarian follicles in all studied groups at the first sampling point

	Control A	Control B1	Control B2	CTX A	CTX B1	CTX B2	P value
E2(ng/L) n=12 rats	68.66 ± 6.65	60.68 ± 5.93	62.34 ± 1.98	35.21 ± 3.93	35.44 ± 4.13	34.71 ± 2.85	<0.005
FSH(IU/L) n=12 rats	7.46 ± 1.71	7.89 ± 1.87	8.16 ± 1.28	19.15 ± 2.43	19.52 ± 1.07	20.04 ± 1.80	<0.005
TNF-α *n* = 6rats	34.67 ± 4.54	94.08 ± 7.42	89.38 ± 7.48	612.80 ± 145.37	609.50 ± 105.37	640.55 ± 55.29	<0.005
No. of primordial and primary follicles	3.17 ± 1.17	3.17 ± 1.17	3.67 ± 3.27	3.50 ± 1.05	3.33±.52	3.33±.52	>0.005
No. of secondary and antral follicles	2.83±.75	2.67±.82	2.67 ± 1.75	1.00±.89	1.17±.75	1.00±.89	<0.005
No. of mature follicles	4.17 ± 1.17	3.17±.98	3.33 ± 1.86	1.00±.89	1.17±.75	1.00±.63	<0.005

FSH, E2 and vaginal smear during (monit-2) →sacrificing to evaluate ovarian histopathology and the level of IGF-1 in (sp-2).

2.11 | CTX group B2: (Figure 4)

Conditioning period for 1 week →induction of ovarian failure by cyclophosphamide during (injection-1) period→ monitoring for FSH,E2 and vaginal smear during (monit-1) →sampling and sacrificing of 6 rats in (sp-1) for ovarian histopathology and level of TNF-α→ receiving Iv MSCs injection once during (injection-2) stage→ 2nd monitoring for FSH,E2 and vaginal smear during (monit-2) →sacrificing to evaluate ovarian histopathology and the level of IGF-1 in (sp-2).

3 | STATISTICAL ANALYSIS

Data were coded and entered using the statistical package SPSS version 21. Data were summarized using mean ± standard deviation for the quantitative variable and frequencies (number of cases) and relative frequencies (percentages) for categorical variables. Comparisons between subgroups within each main group, as well as, between CTX subgroups and their corresponding control subgroups were performed as needed and were done using analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than 2 groups and unpaired T test when comparing 2 groups in normally distributed quantitative variables while non-para metrical Kruskal- Wallis test and Mann- Whitney test were used for non-normally distributed quantitative variables (Chan, 2003). For comparing categorical data, Chi square (χ^2) test was performed. Exact test was used instead when the expected frequency is less than 5. Correlations were done using Spearman correlation coefficient. P-values less than 0.05 were considered as statistically significant.

4 | RESULTS

4.1 | Monitoring 1

4.1.1 | Regarding estrogen level at the first sampling point

All rats in subgroups (CTX A, CTX B1, and CTX B2) which have received cyclophosphamide injections, showed a significant decrease ($p < 0.05$) in the mean value of the serum levels of E2, by the end of a week after the last cyclophosphamide injection, as compared to rats in all control subgroups which subjected to either repeated saline injections (control B1 and B2) or no injection (control A).There was also a significant decrease in the mean value of serum E2 in control B1 (60.68 ± 5.93) and B2 (62.34 ± 1.98) subgroups as compared to control A subgroup ($p < 0.05$) (Table 2).

4.1.2 | Regarding FSH levels at the first sampling point

The mean value of the serum levels of FSH levels was significantly increased ($p < 0.05$) in subgroups (CTX A, CTX B1 and CTX B2) (19.15 ± 2.43, 19.52 ± 1.07 and 20.04 ± 1.80 respectively) as compared to the corresponding value in all control subgroups (Control A, B1 and B2) (7.46 ± 1.71, 7.89 ± 1.87 and 8.16 ± 1.28 respectively) ($p < 0.05$) (Table 2).

4.1.3 | Regarding TNF-α levels at the first sampling point

The mean value of the ovarian tissue homogenate levels of TNF-α was significantly increased in subgroups (CTX A, CTX B1 and CTX B2) (618.8 ± 145.37, 609.5 ± 105.37 and 640.55 ± 55.29 respectively) as compared to all control subgroups (Control A, B1 and B2) (34.67 ± 4.54, 94.08 ± 7.42 and 89.38 ± 7.48 respectively) ($p < 0.05$) (Table 2).

4.1.4 | Regarding the cytological vaginal smear evaluation

All rats in control subgroups exhibited regular 4-5 day cycles throughout monitoring-1 period with regular appearance of cornified cells indicating estrous phase and denoting normal estrogenic activity, in contrast to, CTX subgroups showed marked irregularity in estrous cycles of all rats during the week of monitoring −1 period, with no detection of cornified cells in vaginal smears of 34/36 (94.4%) of rats in all CTX subgroups.

4.1.5 | Regarding of histological studies

All chemotherapy subgroups (CTXA, CTXB1, and CTXB2; 1.00 ± 0.89, 1.17 ± 0.75, and 1.00 ± 0.89 respectively)showed a significant decrease ($p < 0.05$) in the mean value of the number of secondary antral follicles as compared to control A (2.83 ± 0.75) and control B1 (2.67 ± 0.82) subgroups, there also showed insignificant decline of the mean value of the number of primordial and primary follicles (CTXA, CTXB1, and CTXB2; 3.50 ± 1.05, 3.33 ± 0.52, and 3.33 ± 0.52, respectively) as compared to all control subgroups (3.17 ± 1.17, 3.17 ± 1.17, and 3.67 ± 3.27). And a significant decrease ($p < 0.05$) in the mean value of the number of mature follicles and the total number of follicles (CTXA, CTXB1, and CTXB2; 1.00 ± 0.89, 1.17 ± 0.75, and 1.00 ± 0.63,

TABLE 3 Comparison between ovarian architecture scores of each CTX subgroup with its corresponding control subgroup respectively at the first sampling point

Score			Control A	CTX A	*P* value
Architecture score	2	Count	0	6	0.002
		(%)	(0%)	(100.0%)	
	5	Count	6	0	
		(%)	(100.0%)	(0%)	
			Control B1	**CTX B1**	***P* value**
Architecture score	2	Count	0	6	0.002
		(%)	(0%)	(100.0%)	
	4	Count	6	0	
		(%)	(100.0%)	(0%)	
			Control B2	**CTX B2**	***P* value**
Architecture score	2	Count	0	6	0.002
		(%)	(0%)	(100.0%)	
	4	Count	6	0	
		(%)	(100.0%)	(0%)	

respectively as compared to all control subgroups (4.17 ± 1.17, 3.17 ± 0.89, and 3.33 ± 1.86) (Table2).

4.1.5 | Regarding the assessment of ovarian architecture

All rats in CTX subgroups which have received cyclophosphamide injections, showed a significant decrease (*p* value 0.002) in their ovarian architecture scores as compared to all rats of its corresponding control subgroups subjected to either repeated saline injections (control B1 and B2) or no injection (control A) (Table 3).

4.2 | Monitoring 2

4.2.1 | Regarding estrogen and FSH levels at the second sampling point

By the end of the 2nd week following single MSCs, saline or no injection according to the subgroup (Table 1), all rats in chemotherapy subgroups (CTX A, CTX B1, and CTX B2; 30.82 ± 2.83, 29.88 ± 2.83, and 43.55 ± 1.94, respectively) still showed a significant decrease in the mean value of the serum levels of E2 (30.82 ± 2.83, 29.88 ± 2.83, and 43.55 ± 1.94, respectively) ($p < 0.05$) as compared to the corresponding values in all control subgroups (70.85 ± 5.42, 63.5 ± 5.58, and 64.84 ± 2.88 respectively). All rats in chemotherapy subgroups (CTX A, CTX B1, and CTX B2; 20.3 ± 2.06, 20.08 ± 1.6, and 13.12 ± 1.41, respectively) still showed a significant increase in the mean value of the serum levels of FSH(20.3 ± 2.06, 20.08 ± 1.6, and 13.12 ± 1.41, respectively) ($p < 0.05$) in the mean value of the serum levels of FSH, as compared to rats in all control groups (6.28 ± 0.87, 7.55 ± 1.36, and 8.74 ± 1.91, respectively) (data not shown).

Also, by comparing the mean value of the serum levels of E2 and FSH in CTX B2 subgroup which has received single MSCs injection to the corresponding values in both CTX A and CTX B1 subgroups (which has received no injection and single saline injection respectively), it was found that, there was a significant increase ($p < 0.05$) in the mean value of the serum levels of E2 and a significant decrease ($p < 0.05$) in the mean value of the serum levels of FSH in CTX B2 subgroup as compared to CTX A and CTX B1 subgroups (data not shown).

4.2.2 | Regarding the histological assessment at the second sampling point

Homing of MSCs labeled with iron oxide particles into the ovarian tissue, was evident using Prussian blue staining. The histological findings revealed MSCs entrapment in ovarian stroma rather than ovarian follicles. Both CTX A and CTX B1 subgroups showed complete absence of all types of ovarian follicles, while CTX B2 subgroup which has received single MSCs injection showed insignificant difference ($p > 0.05$) in the different types of follicles as compared to all control subgroups (Table 4).

4.2.3 | Regarding the assessment of ovarian architecture of all studied groups at 2nd sampling point

Rats in CTX A and CTX B1 subgroups showed a significant decrease (*p* value 0.002) in their ovarian architecture scores as compared to all rats of its corresponding control subgroups respectively. While rats in CTX B2 subgroup which has received single MSCs injection showed no significant difference (*p* value 1) in their ovarian architecture scores as compared to control B2 subgroup (Table 5).

4.2.4 | Regarding the immunohistochemical expression of IGF-I at the second sampling point

Rats in subgroups CTX A and CTX B1 showed a significant decrease (*p* value 0.002) in the expression of IGF-I as compared to all rats of their corresponding control subgroups, respectively. While rats in CTX B2 subgroup which has received single MSCs injection showed no significant difference (*p* value 1) in its expression compared to control B2 subgroup.

By comparing of both control B2 and CTX B2 subgroups with control A subgroup we found a significant increase (*p* value 0.002) in ovarian IGF-I expression in control B2 and CTX B2 subgroups as compared to control A subgroup.

4.2.5 | Regarding the serum levels of E2 (ng/L) and FSH (IU/L) in all studied groups over the time line period of the experimental protocol

There was a significant decrease in the mean value of the serum levels of E2 in CTX B1 and FSH in CTX B2 subgroup at the end of the 2nd week of monitoring -2 period [M2 (a)], as compared to the corresponding value of the same subgroup at the end of the week of the monitoring-1 period. Also, there was a significant increase in the mean value of the serum levels of E2 in CTX B2 and a significant decrease in the mean value of the serum levels of FSH in CTX B2 at the end of the 8th week of monitoring -2 period [M2 (b)] as compared to the corresponding value of the same subgroup at [M2 (a)] (Table 6).

TABLE 4 The number of ovarian follicles in all studied groups at second sampling point

	Control A	Control B1	Control B2	CTX A	CTX B1	CTX B2	*P* value
No.of primordial and primary follicles	3.17±.75	3.17±.75	2.67±.52	.00*	.00!	3.00 ± 1.55+#	· <0.05 between cont A and CTX A. · <0.05 between cont B1 and CTX B1 · >0.05 between Cont B2 and CTX B2 · <0.05 between CTXB2 and both CTXA and B1
No.of secondary and antral follicles	3.17±.98	2.33 ± 1.37	3.17±.75	.00*	.00!	2.67±.52+#	· <0.05 between cont A and CTX A. · <0.05 between cont B1 and CTX B1 · >0.05 between Cont B2 and CTX B2 · <0.05 between CTXB2 and both CTXA and B1
No.of mature follicles	4.00±.63	3.00±.63	2.83±.98	.00*	.00!	3.17±.75+#	· <0.05 between cont A and CTX A. · <0.05 between cont B1 and CTX B1 · >0.05 between Cont B2 and CTX B2 · <0.05 between CTXB2 and both CTXA and B1
Total number of follicles	10.33 ± 1.75	8.50 ± 1.05	8.67 ± 1.63	.00*	.00!	8.83 ± 1.33+#	· <0.05 between cont A and CTX A. · <0.05 between cont B1 and CTX B1 · >0.05 between Cont B2 and CTX B2 · <0.05 between CTXB2 and both CTXA and B1

*Statistically significant compared to corresponding value in control A subgroup ($p < 0.05$).
!Statistically significant compared to corresponding value in control B1subgroup ($p < 0.05$).
$Statistically insignificant compared to corresponding value in control B2subgroup ($p > 0.05$).
#Statistically significant compared to corresponding value in CTX A and CTX B2 subgroup ($p < 0.05$).

5 | DISCUSSION

The capacity of MSCs to ameliorate ovarian dysfunction injury in the chemotherapy model of POF, may be mediated through many mechanisms, such as transdifferentiation to tissue-specific cells, fusion with the existing native cells to improve the organ function by contributing their own genetic and cellular material (Wang et al., 2013) or via the paracrine mediators secreted by MSCs, which may be involved in the repair process (Afifi and Reyad, 2013).

In this study, homing of MSCs labeled with iron oxide particles into the ovarian tissue, was evident using Prussian blue staining. The histological findings revealed MSCs entrapment in ovarian stroma rather than ovarian follicles. Our histological finding support the hypothesis that, MSCs don't differentiate into oocytes or granulosa cells, but they may restore the ovarian function via playing important accessory roles in the microenvironment surrounding the oocytes in the ovary. In several studies, it was established that, MSCs secrete a wide array of cytokines in vitro including VEGF, IGF-I, HGF (Afifi and Reyad, 2013), IL-8, G-CSF, SCF, IL-11, IL-15, IL-10, and bFGF (Choi et al., 2010).

In the current work, immunohistochemical study revealed that, the expression of IGF-I in ovarian tissue was significantly higher in

TABLE 5 Comparison between ovarian architecture scores of each CTX subgroup (CTX A, B1 and B2) with its corresponding control subgroup (Control A, B1 and B2) respectively, at 2nd sampling point

Score			Control A	CTX A	P value
Architecture score	1	Count	0	6	0.002
		(%)	(0%)	(100.0%)	
	5	Count	6	0	
		(%)	(100.0%)	(0%)	
			Control B1	**CTX B1**	**P value**
Architecture score	1	Count	0	6	0.002
		(%)	(0%)	(100.0%)	
	4	Count	6	0	
		(%)	(100.0%)	(0%)	
			Control B2	**CTX B2**	P value
Architecture score	4	Count	6	6	1
		(%)	(100.0%)	(100.0%)	

subgroups which received MSCs injection, as compared to the expression of IGF-I in ovarian tissue of control A. On the other hand, both CTX A and CTX B1 subgroups also showed marked decrease of the expression of IGF-I in their ovaries when compared to control subgroup, denoting positive correlation between IGF-I and architecture score. This result is in agreement with a study of Wandji et al. (1992) who studied the expression of mouse ovarian IGF system components during follicular development and atresia. Results of that study have shown that, the IGF-I transcripts were low in atretic follicles and they suggested that, the decrease in IGF-I mRNA levels is likely a consequence, rather than a cause of granulosa cell apoptosis.

IGF-I seems to play an important role in MSCs restorative effect; First, IGF-I prevents cell apoptosis and promotes functional recovery as suggested by Xu et al. (2007) who denoted that, IGF-I and other paracrine mediators such as VEGF, bFGF directly up-regulated Bcl-2 protein in cardiomyocytes. The homodimers of Bcl-2 may stabilize the mitochondrial membrane and prevent the activation of downstream apoptotic signaling. Also, anti-apoptotic growth factors were suggested to explain how MSCs repair chemotherapy damaged ovaries. Such factors include two crucial growth factors that play a role in the interaction of oocyte and granulosa cells, namely Growth Differentiation Factor-9 (GDF-9) derived from oocyte and Kit-Ligand (KL) produced from granulosa cells (Hendarto et al., 2013).

TABLE 6 Serum levels of E2 (ng/L) and FSH (IU/L) in all studied groups over the time line period of the experimental protocol

		At the end of M1 ($n = 12$)	At M2(a) ($n = 6$)	At M2(b) ($n = 6$)
E2 (ng/L)	**Control A**	68.66 ± 6.65	70.85 ± 5.42	70.37 ± 5.77
	Control B1	60.68 ± 5.93	63.50 ± 5.58	65.77 ± 6.60
	Control B2	62.34 ± 1.98	65.84 ± 2.88	65.15 ± 5.94
	CTX A	35.21 ± 3.93	30.82 ± 2.83	31.67 ± 2.78
	CTX B1	35.44 ± 4.13	29.88 ± 2.83*	29.30 ± 4.56
	CTX B2	34.71 ± 2.85	43.55 ± 1.94*	54.05 ± 6.39**
FSH (IU/L)	**Control A**	7.46 ± 1.71	6.28±.87	6.25±.94
	Control B1	7.89 ± 1.87	7.55 ± 1.36	8.17±.99
	Control B2	8.16 ± 1.28	8.74 ± 1.91	8.67 ± 1.22
	CTX A	19.15 ± 2.43	20.30 ± 2.06	21.18 ± 2.36
	CTX B1	19.52 ± 1.07	20.08 ± 1.60	21.26 ± 1.34
	CTX B2	20.04 ± 1.80	13.12 ± 1.41*	10.23 ± 1.42**

*Statistically significant compared to corresponding value in M1 ($p < 0.05$).
**Statistically significant compared to corresponding value in M2(a) ($p < 0.05$).

Second, in addition to the suggested antiapoptotic role of of IGF-I secreted by MSCs, it might also possess effective anti-oxidative abilities. Baregamian et al. (2006) and Xu et al. (2007) indicated that, the cytokines released from BM- MSCs, including IGF-I, result in the overexpression of Bcl-2 in cardiomyocytes, which also increased their resistance to oxidative stress.

Third, IGF-I may act to ameliorate the damaged ovaries via angiogenic support, since the re-establishment of blood supply is fundamental for recovery of damaged tissues; this was proved by Han et al., (2003), who indicated that IGF-I is a potent angiogenic agent for fetal lung endothelial cells in vitro; Also, IGF-I has been shown to be a potent in vivo angiogenic growth factor in promoting rabbit corneal angiogenesis (Grant et al., 1993).

A recent study conducted by Lai et al. (2014), concluded that, the mechanisms of improvement of ovarian functions after skin derived MSCs (SMSCs) injection not only involve the migration and engraftment of stem cells in host ovaries, but are also likely related to the anti-inflammatory properties of the cells. Their conclusion was based on increase of expression of several pro-inflammatory cytokines, such as TNF-α, IL-8, and IL-6 in the chemotherapy damaged ovaries. These cytokines were significantly decreased after SMSC transplantation in chemotherapy induced ovarian failure group than in untreated mice. In this way, SMSC transplantation can mitigate inflammatory cytokine activity and modulate the inflammatory response in the ovaries. Moreover, the transplanted cells or factors released from the transplanted cells could repair the gonadal microenvironment and improved the conditions for development of germ cells. In this way, SMSCs which were found in the stroma of the ovarian tissue, might differentiate into stroma cells, reduce the inflammatory response in the ovaries, and improve the germ cell niche, which is important for folliculogenesis and fertility (Lai et al., 2014). Other studies have suggested that, the predominant role of MSCs in resolving tissue damage relies on their role in modulation of the inflammatory process (Donizetti-Oliveira et al., 2012).

The attraction of the injected MSCs towards both damaged ovaries by cyclophosphamide, as well as, ovaries of saline subjected animals might be explained by an elevation of ovarian TNF-α levels in both groups as compared to control A subgroup. This finding was also proved by many studies: Chen et al. (2003) suggested that, TNF-α production during acute myocardial injury may help the heart to recruit extracardiac embryonic stem cells, and regenerate damaged myocardium. This suggestion was supported by the previous finding of Min

et al. (2002) which denoted that, stem cells transplantation produced a greater improvement of cardiac function in pigs with acute myocardial infarction than with chronic infarction, which might result from more TNF-α production during the acute stage of infarction. Hemeda et al. (2010) have also confirmed that TNF-α is a potent regulator of MSCs migration in vitro. A recent study by Uchibori et al. (2013) also suggest that, the TNF-α available at the site of injury was proved to stimulate MSCs production of anti-inflammatory factors in vitro. So, TNF-α may help in both migration and homing of MSCs toward the injured tissue, as well as induction of its curative effect.

6 | CONCLUSION

Intravenous injection of BM-MSCs could migrate to home in the damaged ovarian stroma. These MSCs could partially ameliorate hormonal function, folliculogenesis and architecture of chemotherapy damaged ovaries of albino rats used in our study.

Also, the repeated doses of cyclophosphamide that induce ovarian failure are associated with elevated level of TNF-α in ovarian homogenate. This elevated level of TNF-α might have a role in attraction of MSCs toward the damaged ovaries. The elevated level of IGF-I in ovaries of rats receiving MSCs injection, might play a role in MSCs effect on restoration of ovarian function.

Further studies are required to identify different mechanisms involved in stem cells homing; other growth factors and cytokines involved in MSCs mediated repair of the damaged tissues as well as the fate of the produced oocytes from the treated ovaries. This field of research can offer a strong hope for patients with premature ovarian failure.

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