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Full Length Research Paper

Exosomes Derived from Bone Marrow Mesenchymal Stem Cells Restore Cisplatin Induced Ovarian Damage by Promoting Stem Cell Survival, Meiotic, and Apoptotic Markers

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Exosomes released by bone marrow mesenchymal stem cell (BMSCs) provide a novel source and a great potential donor cell for regenerative medicine. The current study was set to explore the restorative potential of BMSCs derived exosomes in a rat model of premature ovarian failure (POF) and its underlying mechanisms. Exosomes were prepared and infused to the cisplatin induced POF rats. The ovaries were subjected for histological and immune-histochemical evaluation of *survivin* and *Oct4*. RNA extraction and quantitative real time PCR were carried out for evaluation of *Stra8, Oct4, Lin28*, and *Nanos3* gene expression. Serum estradiol (E2) and follicular stimulating hormone (FSH) were monitored. The POF group showed reduced number of follicles, excessive fibrosis, follicular atresia, low serum E2 and high FSH levels compared to controls. BMSCs derived exosomes infusion improved ovarian architecture with normal hormonal profile. There was down and up-regulation of *Oct4* and *Lin28*, markers of stemness and germ cell survival, *Stra8* and *Nanos3*, molecules that judge the cellular fate towards meiosis and oocyte formation in POF, and exosomes treated ovaries respectively. This study demonstrate for the first time that exosomes infusion showed structural and functional reparative properties,possibly through delivering its carriage of protein, molecular content and RNA bioactive molecules to the host ovarian niche inducing neo-oogenesis/folliculogenesis. This provides an effective and novel method for treatment of POF.

Keywords: Ovarian failure, Exosomes, *Oct4*, *Lin28, Nanos3, Stra8, Survivin*.

List of Abbreviation

POF, Premature ovarian failure; E2, stradiol; FSH, Follicular stimulating hormones; MSCs, Mesenchymal stem cells; BMSCs, Bone marrow derived mesenchymal stem cells; Stra 8, Stimulated by retinoic acid gene 8; Oct4, Octmar-binding transcription factor; FACS, Flocytometry

INTRODUCTION

Premature ovarian failure (POF) affects women below the age of 40 years, and characterized clinically by irregular menstruation up to its cessation, high Follicular stimulating hormone (FSH), lowestradiol (E2) levels and infertility (Goswami and Conway, 2005). POF confers increased risk for cardiovascular disease osteoporosis, and other menopausal related diseases(Maclaran and Panay 2011). POF has many aetiological factors including genetic, immunological, environmental toxins and drug induced, the later represents 25% of POF causes (Massin et al., 2004). Chemotherapy is the most common form of drug induced POF. The severity of POF depends on the type and combination of drugs, duration of treatment, the use of adjuvant radiotherapy (Chen et al., 2011). Besides the direct effect of chemotherapy on the folliculogenesis via chromosomal breaks, and DNA damage, disturbance of neuroendocrine axis is also involved in early menopausal manifestation and poor fertility outcome in cancer treated females (Mahajan, 2015). The attempts to preserve or restore hormonal profile and fertility following oncologic therapy have always been a concern. Multiple strategies are adopted for preservation of ovarian function in those at risk of POF including, oocyte, ovarian, and embryo cryopreservation (Mahajan, 2015) and of course hormone replacement therapy (Conte and Del Mastro, 2017). However cost, technical, ethical and cultural limitation makes these strategies debatable.

In the era of stem cells, multiple studies reported successful repair of ovarian functions in animal models of POF using bone marrow (Gabr et al., 2016), amniotic membrane (Fouad et al., 2016), amniotic fluid (Xiao et al., 2014), adipose tissue (Su et al., 2016), skin (Lai et al., 2014), endometrial derived stem cells (Lai et al., 2015) and menstrual blood derived stem cells (Liu et al., 2014b). Therapeutic effects of stem cells are not directly related to their homing to the affected organ neither to their differentiation into cellular components of the affected organ. In fact many reports indicated that stem cells are short lived and majority of the injected cells for the purpose of organ regeneration are not engrafted by the affected organs (Lai et al., 2014; Liu et al., 2014a). Currently, it is widely accepted that stem cells exert their therapeutic immuno-modulatory and regenerative actions through paracrine mechanisms involving soluble molecules and subcellular machineries. Stem cells treatment was found to target many pathways, including modulation of inflammatory, angiogenic, and apoptotic gene expression, as well as alteration of genes involved in meiosis and early oocyte development. Extracellular vesicles released by stem cells are suggested, among others, to be responsible for the stem cell induced cell to cell communication (Yuan et al., 2016).

Many types of extracellular vesicles are known, of which exosomes. Exosomes are endosomal derived particles and described to be of 40-100nm diameters and can be separated by centrifugation at 100,000 g of mesenchymal stem cells (MSCs) culture supernatants(Van Deun et al. 2014). Due to DNA, RNA, and proteins contents of exosomes (Abramowicz et al., 2016), they were found to be effective in modulating many pathological conditions such as liver fibrosis, acute kidney injury, myocardial infarction, and ischemia induced neural degeneration, reviewed in (Di Rocco et al., 2016).

The aim of the present study is to illustrate the efficiency of using bone marrow mesenchymal stem cells (BMSCs) derived exosomes in restoration of hormonal profile and folleculogenesis of cisplatin induced rat model of POF. Also evaluate the role of meiotic, oogenesis and apoptotic regulators; *Stra8, Oct4, Nanos3, lin28* and *survivin* as a potential pathway targeted by stem cell derived exosomes.

MATERIALS AND METHOD

Animals

Sexity five mature (12-week old) white albino female rats from an inbred colony (Curl: HEL1), all were matched age and weight were used in the experiments. Their mean weight was 200 g with a range 160–220 g. Animals were allowed to adjust in cages (5 animals per cage) inside a well-ventilated room for 1 week. The animals were maintained under standard laboratory conditions: temperature of 23°C, relative humidity of 60–70%, and a 12-hour light/dark cycle. The rats were fed a standard pellet diet and allowed free access to water. Vaginal smears were obtained daily. Only rats showing at least two consecutive normal 4 to 5-day vaginal estrus cycles were included in the experiments. All animal procedures were performed in accordance with guidelines for the care and use of experimental animals as approved by the Animal Ethics Committee, Cairo University.

Isolation, phenotype and characterization of BMSCs

BMSCs were acquired from the 10-week-old rats (n = 10). Bone marrow was flushed out from tibias using phosphate buffer saline (PBS; Gibco/Invitrogen, Grand Island, New York, USA) and centrifuged at 1,000 rpm for 5 min. BMSCs were cultured with a RPMI medium (Gibco BRL, USA), 10% fetal bovine serum (FBS, Gibco BRL, USA), and maintained in a cell culture incubator containing 5% CO2 at 37ºC. At 80-90% BMSCs confluence, they were detached with 0.25% trypsin-EDTA (Gibco BRL, USA), and sub-cultured in new flasks. Third passage BMSCs were used in all experiments. BMSCs were characterized in culture by their morphological spindle shaped like cells. Further identification of BMSCs was established by flow cytometry (FACS) (Beckman Coulter). BMSCs were suspended (1x 10^6 cells/ml) and stained with FITC conjugated monoclonal antibodies, CD29 (Biolegend), CD105 (Biolegend), and CD34 (Biolegend)(Liu et al. 2014a).

Isolation and identification of exosomes

Exosomes were obtained from supernatants of third passage BMSCs (5x10^6cells /ml) cultured in RPMI deprived of FBS and supplemented with 0.5% of bovine serum albumin (BSA) (Sigma). After centrifugation at 2000 g for 20 min to remove debris, cell-free supernatants was centrifuged at 100,000 g (Beckman Coulter Optima L 90K ultracentrifuge) for 1 h at 4°C, washed in serum-free medium 199 containing HEPES 25mM (Sigma) and submitted to a second ultracentrifugation in the same conditions. Electron microscopy analyses of exosomes were performed. Purified exosomes cultured overnight in the medium used for collection of exosomes. Images were obtained by electron microscope at a working distance of 15 to 25 mm and an accelerating voltage of 20 and 30 kV. Digital acquisition and analysis were performed using the Jeol T300 system. FACS analysis was performed using FITC-conjugated antibodies: CD83 (MiltenyiBiotec) and CD73 (Becton Dickinson). FITC mouse non immuneisotypicIgG (DakoCytomation) was used as control (Gatti et al. 2011).

POF model and intravenous injection of exosomes

The animals were divided into three main groups (12 rats/group): normal control group (Controls), cisplatin-induced POF group (POF) and BMSCs derived exosomes treatment group (Exosomes). The POF rat model was established by intraperitoneal injection with a daily dose of cisplatin (in DMSO) at 2 mg/kg of body weight for six days. POF was confirmed by histological examination of ovaries of 5 rats. Exosomes (derived from 5 x 10^6 BMSCs/rat) suspended in 0.6 mlDMSO and were injected via the tail vein on the seventh day. Additionally, two other groups(12 rats/group): normal and POF rats were injected with DMSO at the specified times and served as a vehicle controls. At day 15 and 30, six rats from each group along were narcotized; the blood and ovaries were collected. The left ovaries were dissected immediately; thirty mg were snap frozen to be used for RNA extraction, and other parts were fixed in 10% paraformaldehyde for histological and immune-histochemical studies(Liu et al. 2014b).

Hormonal assay

Blood was collected from retro-orbital vein. After collection of the whole blood in a plain red topped tube, blood was allowed to clot for 30 minutes, then centrifuged at 4000 g for 20 minutes, serum was collected and stored in Eppendorf tubes and stored at -20°C for hormonal. The level of E2 and FSH were assessed with a CUSABIO Reagent Kit (USA).

Ovarian Morphologic Analysis and Immunostaining

After 15 and 30 days the left ovary specimens were fixed in 10% buffered formalin solution for 48 h, dehydrated in ascending grades of ethanol, and embedded in paraffin. Serial sections of 5–7 µm thickness were cut, mounted on glass slides, and subjected to haematoxylin and eosin (H&E) staining for histological assessment (Kiernan, 1999), Masson Trichrome (Bancroft, 2008) and Immuno-histochemical staining for *survivin* and *Oct4,* using the avidin–biotin–peroxidase complex. For the purpose of follicular counting, serial sections stained with H&E were morphometrically analysed using a Leica Quin500C image analyzer computer system (Leica Imaging System Ltd., Cambridge, England). The maximum diameter and diameter at the right angle were used to obtain a mean diameter for each follicle (Abd-Allah et al., 2013).

For the purpose of immune-staining, ovarian sections were incubated with rabbit polyclonal antibodies and counterstained with Meyer's hematoxylin. The antibodies were purchased from Gentex (Irvine, California): rabbit polyclonal antibody to *survivin* catalogue# GTX100052 and to *Oct4*, catalogueC GTX59610. Antibodies were used at a dilution of1:50 and 1:200, respectively. *Survivin*-positive cells showed brown cytoplasmic or nuclear deposits (Jiang et al., 2014). *Oct4*: showed positive brown reaction in granulosa cells and interstitial tissue of normal ovary while negative reaction observed in tissue of damaged ovaries where the reaction was restricted to stromaonly (Liu et al., 2014a). Negative controls were obtained by processing additional ovary specimens in the same way but skipping the application of the primary antibody (Santa Cruz Biotechnology Inc.). Morphometric analysis was carried out using a Leica Qwin 500 image analysis computer system (Leica Microsystems Ltd, Cambridge, UK) the following

parameters were measured: Mean area% of positive immuno reactivity for *survivin* and *Oct4.* Ten high-power fields were observed for each specimen at a magnification of ×400.

Quantitative real time PCR

Ovarian tissue of all studied groups was homogenized and total RNA was isolated with RNA easy Mini Kit (Qiagen, USA). RNA sample were further analyzed for quantity and quality with Beckman dual spectrophotometer (USA). cDNA was prepared by reverse transcriptase kit (#K1621, Fermentas, USA). qRT-PCR for *Oct4, Stra8, Lin28* and *Nanos3* genes expression was performed. The relative expression of the mRNA was assessed using StepOne Real-Time PCR Systems (Applied Biosystems, Foster City, CA,USA). The PCR primers used were demonstrated in Table 1. Quantitative RT-PCR analyses were performed in 25 µl total reaction volume consisting of 12.5µL SYBR Green PCR master mix (Applied Biosystems, USA), 1000 nM of each primer, and 5 µL of cDNA after 1/5 dilution. The amplification thermal profile was, 10 min at 95 ºC, and 40 cycles of denaturation at 95 ºC for 30 s and annealing/extension at 60 ºC for 1.5 min. Data from the real-time assays was calculated by Software version 1.7 (PE Biosystems, Foster City, CA, USA). The relative expression levels of *Oct-4, Stra8 Lin28* and *Nanos3* were calculated by the delta CT method according to the manufacturer recommendations (Applied Biosystems, USA) with using GAPDH as house-keeping gene.

Statistical analysis

The statistical differences between experimental groups were determined by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons or unpaired t-test for single comparison of experimental data to the control using GraphPad Prism analysis software. Results were considered significant at $P < 0.05$.

RESULTS

Characterization of BMSCs and exosomes

Cultured third passage of BMSCs were characterized using inverted microscope by their morphological fibroblast like cells (Figure 1A). They were acquired both positive high expression of CD29 (99.82%) CD 105 (96.98%) and negative expression of CD34 + (0.02%) (Figure 1B). Exosomes were analysed by electron microscopy that represented micrographs for spheroids exosomes with diameter less than 100nm (Figure 2A). FACS analysis of exosomes showed their acquiring surface markers such as CD83 (99.67%) and CD73 (99.9%) (Figure 2B).

Serum levels of FSH and E2

Levels were analysed 15 and 30 days after injection of exosomes. There was a statistically significant difference between groups p = 0.0047 and P= 0.002) after 15 and 30 days of exosomes injection respectively. A Tukey post-hoc test revealed that after 15 days, FSH levels were statistically significantly higher in cisplatin induced ovarian failure (93.83 ± 10.99 mIU/ml) compared to the control group (39.83 ± 5.76mIU/ml) $P < 0.01$, while there were no statistically significant differences between the exosomes treated group (62.33±11.66 mIU/ml) and POF or control groups ($P >0.05$) at this time point. Contrary, after 30 days of treatment, FSH levels were lowered in bothtreated group (59.67 ± 3.79 mIU/ml) and control (41.83 ± 3.87 mIU/ml) compared to POF group (97.67 ± 14.52 mIU/ml); $P <0.01$) and $P<0.05$ respectively, (Figure 3A). E2 showed significant reduction after 15 (15.40±0.45 ng/ml) and 30 days (13.33±0.61ng/ml) of establishment of POF by cisplatin injection compared to their corresponding controls (49.02±7.62ng/ml, $P <0.001$) and (52.90±5.09ng/ml, $P <0.001$). Injection of exosomes successfully restored the E2 levels back to its normal values (61.55±5.01 ng/ml, $P <0.01$), as early as 15 days post experimental intimation and the improvement was more manifest after 30 days after exosomes injection (65.72±3.49 ng/ml, $P <0.01$) compared to their corresponding untreated POF groups, (Figure 3B).

Histological, morphometric and immunostaining analysis

The control group exhibited normal histological architecture. The cortex contained primordial, primary, secondary and antral follicles as well as corpora lutea and albicans. The germinal epithelium is of simple cuboidal type (Figure 4A and 4B). Fifteen days after induction of the POF model, POF group showed degenerated; surface epithelia, few primordial, secondary, and antral follicles as well as degenerated cortical and medulla areas (Figure 4C). Thirty days post experimental duration ovary tissues showed complete loss of tissue architecture and antral follicles were not observed (Figure 4D). In contrast 15 days after exosomes treatment ovarian sections showed improvement in tissue architecture with well observed primordial follicles and developing surface epithelium (Figure 4E). However, after 30 days of exosomes treatment, all types of follicles were apparently normal and blood vessels showed patent lumen (Figure 4F and G). Follicular count revealed a significant decrease in number of follicular count in POF when compared to controls and exosomes treated group. However the number of antral follicles is significantly higher in exosomes treated group when compared to control group Table 2. Histological evaluation showed an average of

Table 1. The primers' sequence of studied genes

Gene symbol	Primer sequence: 5′-3′	GB accession number
Oct-4	F: CCTGCAGCAGATCACTAGCAT R: ACTCGAACCACATCCCTCT	NM_001009178.1
Stra-8	F:TCATF:TCATCGAGTTTTTCAAAGG R:TCCACAGGAGGATCTGGTTC	XM_001067836.1
Lin-28	F: CGGGCATCTGTAAGTGGTTC R: CAGACCCTTGGCTGACTTCT	NM_024674.5
Nanos3	F: CCGTGCCATCTATCAGTCCC R:AATTCCGGGTGGTGTAGCAG	NM_001105945.1
GAPDH	F:CTCTACTGGCGCTGCCAAGGCT R:GTCCACCACTGCACGTTGG	NT009759.16

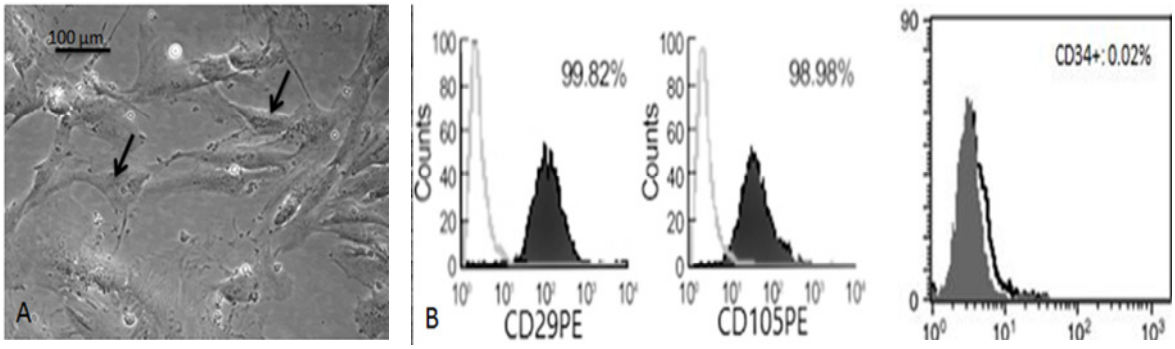

Figure 1. Characterization of MSCs, A) BMSCs were characterized using inverted microscope by their morphological fibroblast like cells, B) They were acquired both positive high expression of CD29 (99.82%) and CD 105 (96.98%).

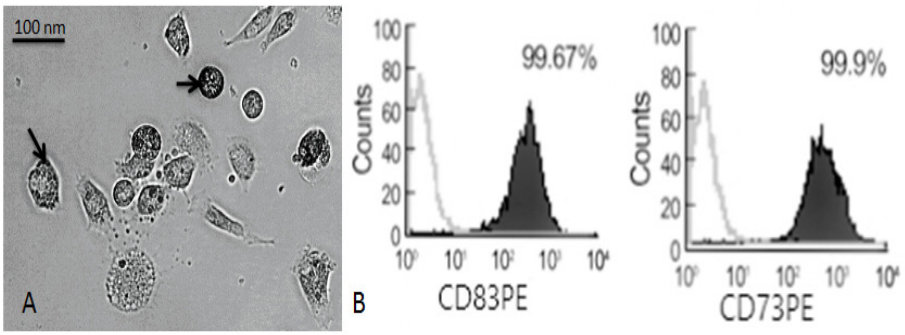

Figure 2. Characterization of MVSC, A) Exosomes were analysed by electron microscopy that represented micrographs for spheroids exosomes, B) FACS analysis of exosomes showed their acquiring surface markers such as CD83 (99.67%) and CD73 (99.9%).

Table 2. Number of small, medium and large ovarian follicles in H&E-stained sections

No. of follicles	Controls	POF-15	POF-30	Exosomes-15	Exosomes-30	$P value
Small follicles	9.2 ± 2.1	16.0± 0.9	15 ±0.8	7.9 ±3.1#	8.9 ± 3.2#	<0.001
Medium follicles	24.9 ± 1.7	5.2 ± 1.3*	4.7 ±1.2*	20.7 ± 0.4#	23.2 ± 0.3#	<0.001
Large follicles	8.0 ± 0.15	1.3 ± 0.9*	1.1 ±0.8*	4.5 ± 0.6#	7.9 ±0.7#	<0.001

*Significance at P < 0.01 compared to controls, # Significance at P < 0.01 compared to corresponding POF group. Data are presented as mean ± SD and $ P value comparing groups using ANOVA.

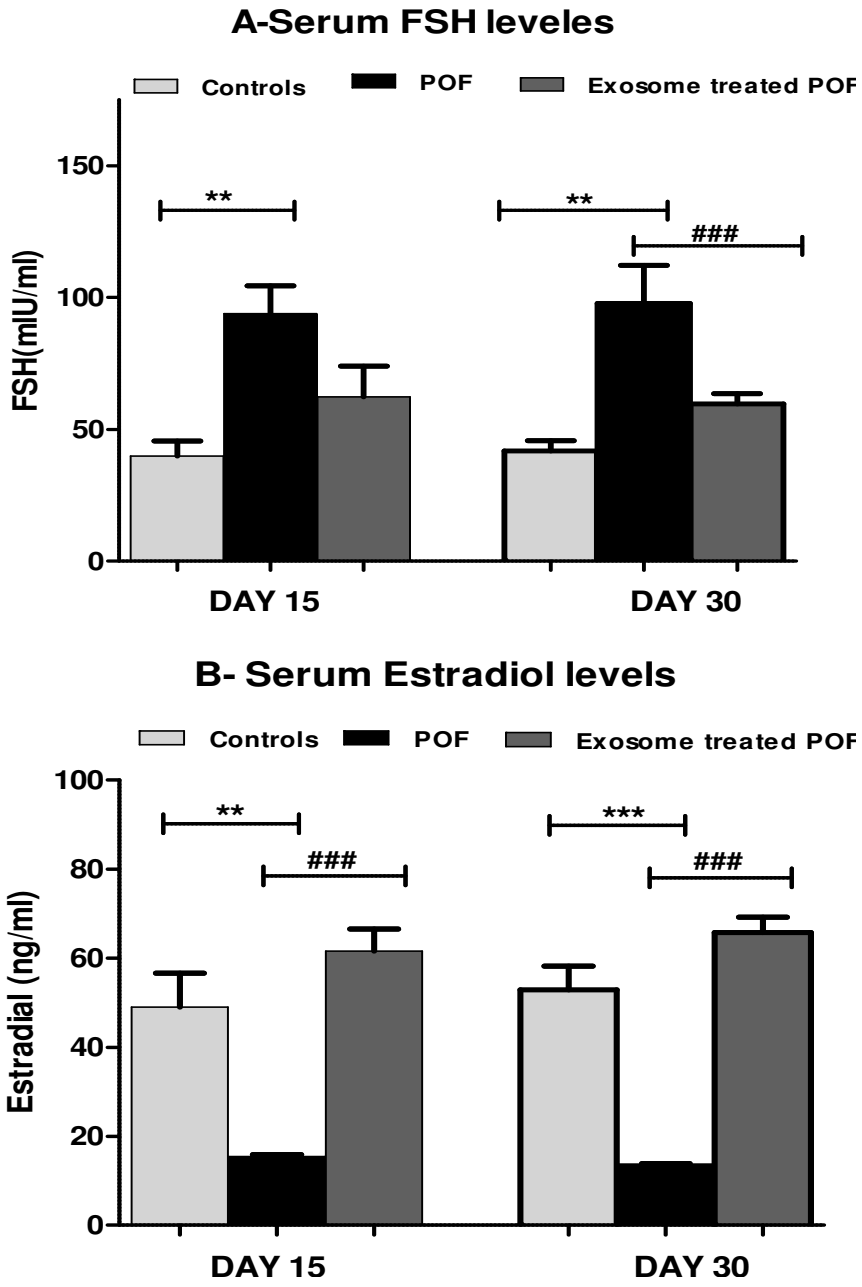

Figure 3. Hormonal profile, A) FSH, B) Estradiol in rat study groups; controls, induced premature ovarian failure (POF), POF treated exosomes (exosomes treated POF) 15 and 30 days after treatment. Data represent the mean ± SD of 6 animals, *Significantly different from respective controls ($P < 0.05$); **($P < 0.01$); ***($P < 0.001$). # significantly different from POF P value < 0.05; ##($P < 0.01$); ###($P < 0.001$)

802 follicles at the antral stage compared to only 632 in untreated controls.

Masson Trichrome Stained ovary sections

Ovarian sections of the control group showed scanty collagen deposition within the cortical stroma between the cortical follicles (Figure 5A). Ovarian sections of POF group showed increased collagen deposition in the cortical stroma among the few maintained follicles, that extending towards the medulla (Figure 5B). On the other hand decreased collagen fibrecontent through the whole ovary tissue and well detected healthy cortical stroma between follicles were observed in exosomes treated groupsa 15 and 30 days (Figure 5C and D).

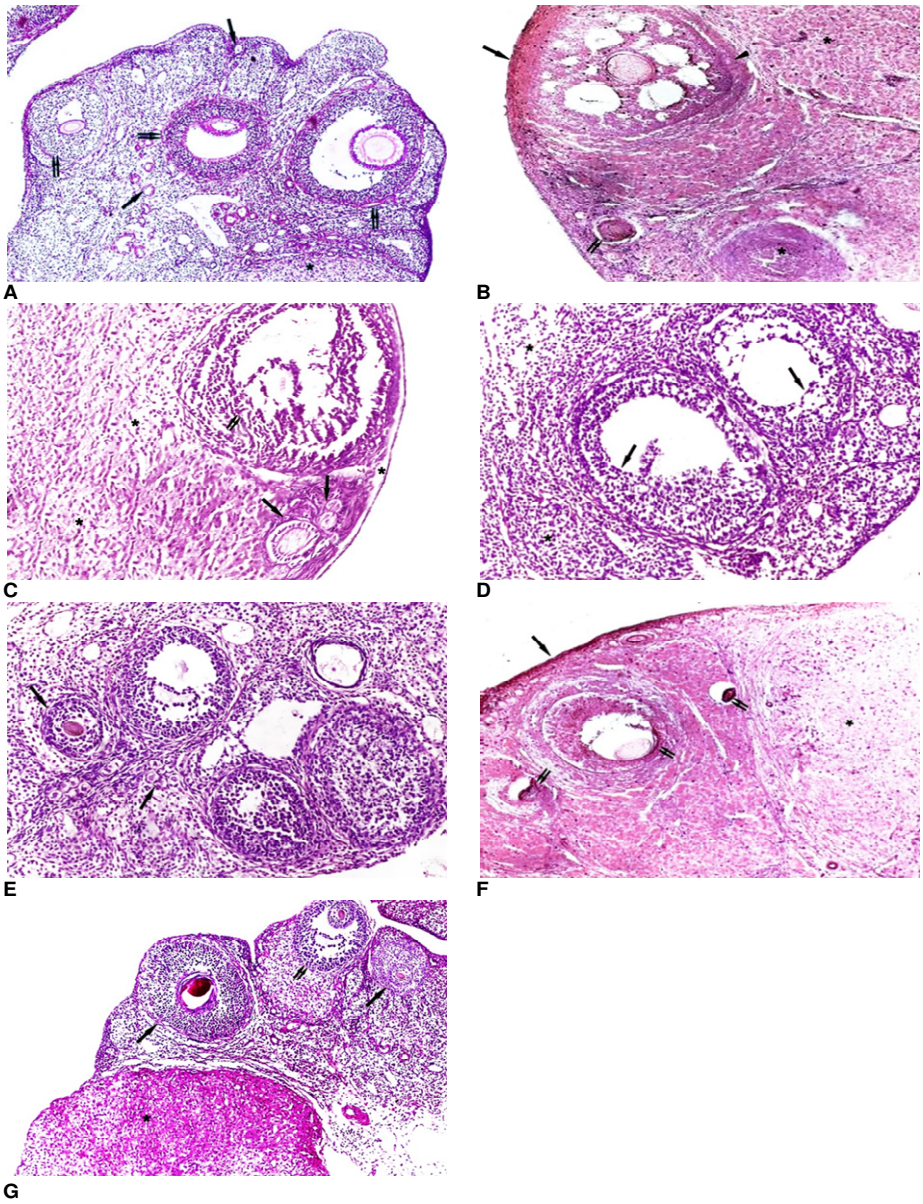

Figure 4. Photomicrograph of H &E; x200 stained ovaries sections A) controls 15 days post experimental duration where cortex contained primordial follicles, primary, secondary and Graafian follicles (arrows), where the medullary region was clearly distinguished by numerous arteries (arrow). B) Controls 30 days post experimental duration, Corpora lutea and albicans were also observed. The germinal epithelium is of simple cuboidal type (arrow). C) POF group 15 days post experimental duration showing degenerated surface epithelia, primordial follicles with incomplete growth (arrows), few primary oocytes were still observed (arrows). D) POF group 30 days post experimental duration showing absence of developing follicles (arrows), corpus luteum is almost degenerated (arrow). E) Exosomes treated group 15 days post experimental duration showing well observed primordial follicles with reduced degenerated areas at both cortex and medulla with developing surface epithelium (arrows). F) & G) Exososmes treated group 30 days post experimental duration showing well-observed ovary lining of cuboidal epithelium (arrows), follicles of different types (arrows); corpus luteum were apparently normal and blood vessels (arrow) showing open lumen

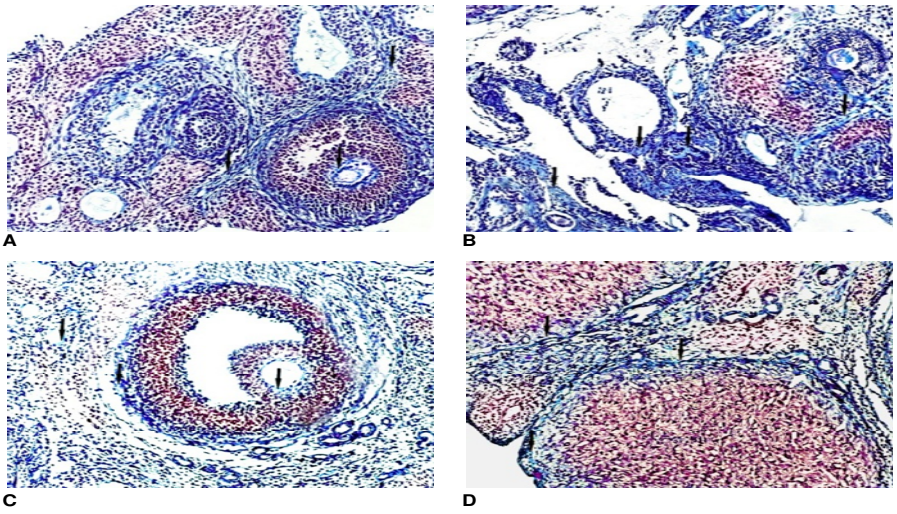

Figure 5. Photomicrograph of Masson trichrome; x200 stained ovaries sections A) Control group showing few collagenous fibers in the ovarian cortical stroma between the cortical follicle (arrows). B) POF group 30 days post experimental duration showing markedly increased collagenous fibers in the ovarian cortical stroma(arrows) between follicles. C) Exosomes treated group 15days post experimental duration shwoing more collagenous fibers than normal and a lot of degenerated areas are still observed (arrows) and D) Exosomes treated group 30 days post experimental duration showing decrease in the collagenous fibers content through the whole ovary tissue (arrows) with well detected healthy cortical stroma.

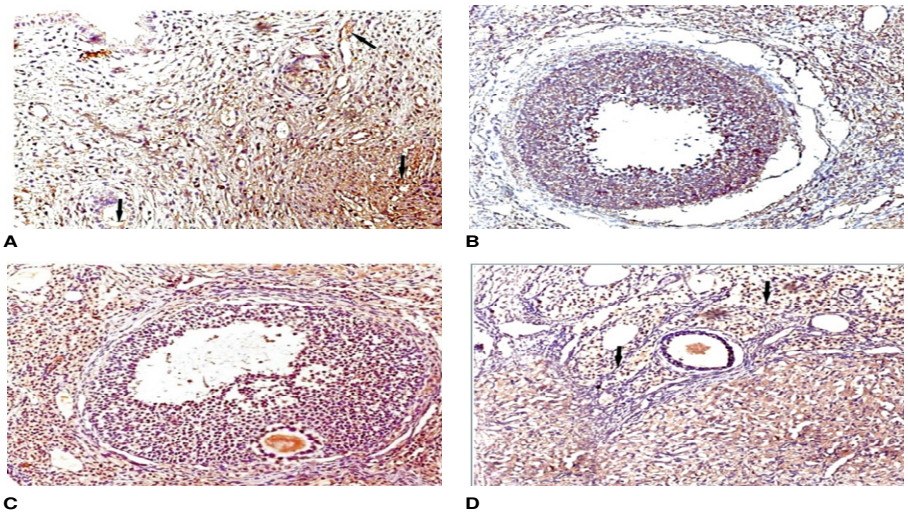

Figure 6. Photomicrograph of *Survivin*immuno-stain; x200. A) Control group 30 days post experimental duration showing positive *survivin*immuno-reactive nuclei (arrows) among all oocytes and granulosa cells, B) POF group 30 days post experimental duration showing negative *survivin*immuno-reactive nuclei, well-observed degenerated cortex and medulla. C) Exosomes treated group 15 days and D) 30 days post experimental duration showing highly increased *survivin*immuno-reaction nuclei.

Survivin and *Oct4* immunostaining

Controls showed highly positive *survivin* immune-reactive nuclei among oocytes and granulosa cells (Figure 6A). There was a marked decrease in *survivin* immuno reactivity in POF group. The surface epithelial as well as primordial cells showed lots of degenerations (Figure 6B). Meanwhile, exosomes treated group showed increased immune-positive reaction of *survivin* in surface epithelial cells, follicular cells as well as oocytes of a primordial follicle15 and 30 days after exosomes infusion (Figure 6C and D).

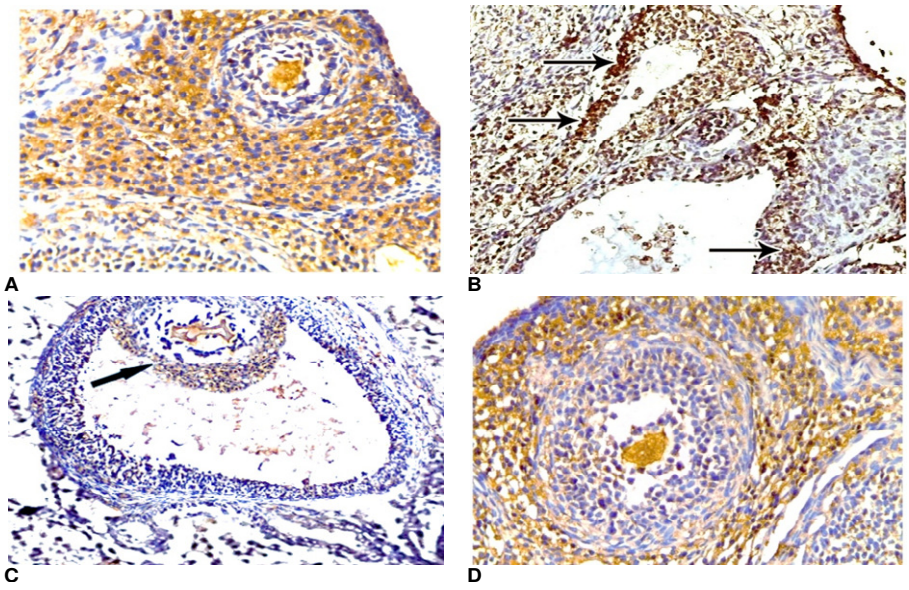

Figure 7. Photomicrograph of *Oct4* immuno-stain; x200. A) control group 30 days post experimental duration showing positive showing positive *Oct4* immuno-staining in cytoplasm of all germ cells and oocyte, B) POF group 30 days post experimental duration showing faint *Oct4* immuno-staining in the cytoplasm of all cells of the ovarian tissue. C) Exosomes treated group 15 days and D) 30 days post experimental duration showing moderate and high *Oct4* immuno-staining in the cytoplasm of all cells of the ovary respectively.

Table 3. The mean area %, SD of *Oct4*, *Survivin* immuno-expression and Masson Trichrome staining in different study groups

	Controls	POF-15	POF-30	Exosomes- 15	Exosomes-30
Oct4	5.03+0.29	1.21+0.27*	1.01+0.81*	5.11+0.39#	3.75+0.42#
Survivin	7.52±0.41	0.52±0.07*	0.32±0.09*	5.40±0.47#	6.38±0.38#
Mason Trichrom	0.17±0.03	5.42±0.59*	6.31±0.87*	0.28±0.08#	0.18±0.07#

*Significance at P < 0.01 compared to controls, # Significance at P < 0.01 compared to corresponding POF group. Data are presented as mean ± SD

A regard *Oct4*, controls showed positive *Oct4* immuno-staining in cytoplasm of all germ cells and oocyte of small growing follicles. Stromal cells showed no *Oct4* immunostaining (Figure 7A). POF group showed very faint *Oct4* immunostaining limited to the observed undamaged germ cells (Figure 7B). Strong *Oct4* immunostaining were observed at fifteen days post exosomes treatment. Where, the cytoplasm of most cells of the ovarian tissues showed high staining of *Oct4* (Figure7C). The *Oct4* staining were reduced to be limited to the observed germ cells and cytoplasm of growing follicles thirty days post experimental duration (Figure 7D).

Table 3 summarized Mason trichrome staining scores, the *survivin,* and *Oct4* Immuno-scores of the different study groups. Where POF showed marked reduction of both *Oct4* and *Survivin* and marked collagen deposition compared to controls and exosomes treated groups (P<0.01). In the mean- time no significant variation between controls and exosomes treated group indicating the success of exosomes treatment to restore ovarian function in the studied rat model.

Gene expression analysis

To understand the mechanism by which of BMSCs derived exosomes administration ameliorated cisplatin induced ovarian failure, qPCR was performed at 15 and30 days after exosomes infusion using primers specific for the meiosis stimulation factor *Stra8* which stimulate the oocyte to enter the 1st meiotic division, stem cell survival and markers for meiotic commitment *Oct4, Lin28*, and oocytes specific transcriptional factor *Nanos3*. Data was expressed as delta Ct change relative to GAPDH. As shown in Figure 8a and b, control ovarian tissues normally express *Sta8, Oct4, Lin28*, and

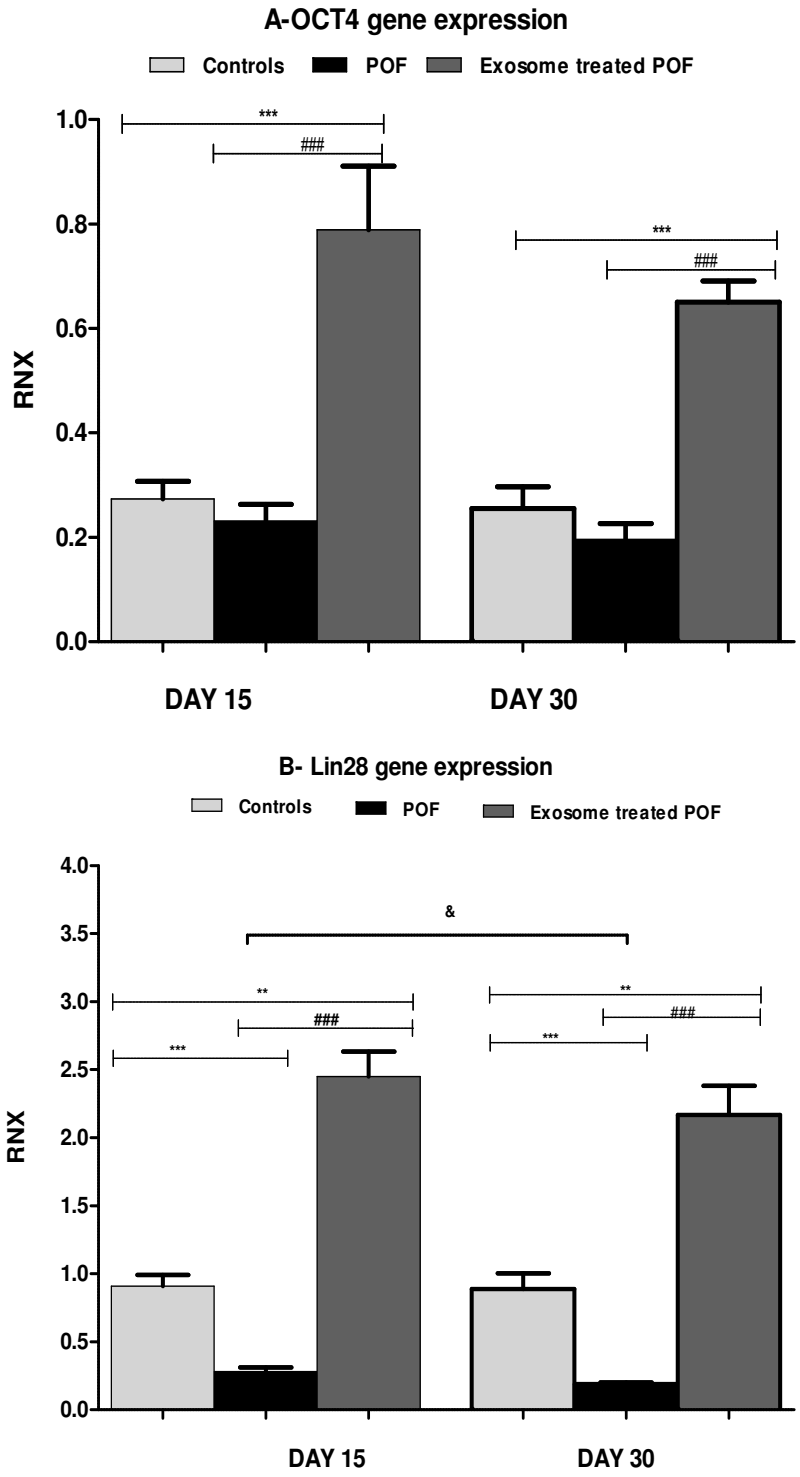

Figure 8 a. Relative gene expressions of A) Oct-4, B) Lin28, in rat study groups; Controls, induced premature ovarian failure (POF), POF treated exosomes (Exosomes treated POF) 15 and 30 days after treatment. Data are expressed as RNX, relative expression (delta CT) where GAPDH was used as internal control gene. *Significantly different from respective controls ($P < 0.05$); **($P < 0.01$); ***($P < 0.001$). # Significant different from POF group (P value < 0.05); ##($P < 0.01$); ###($P < 0.001$), & and ð Significant different between 15 and 30 days of POF and exosomes treated repectively at P <0.5.

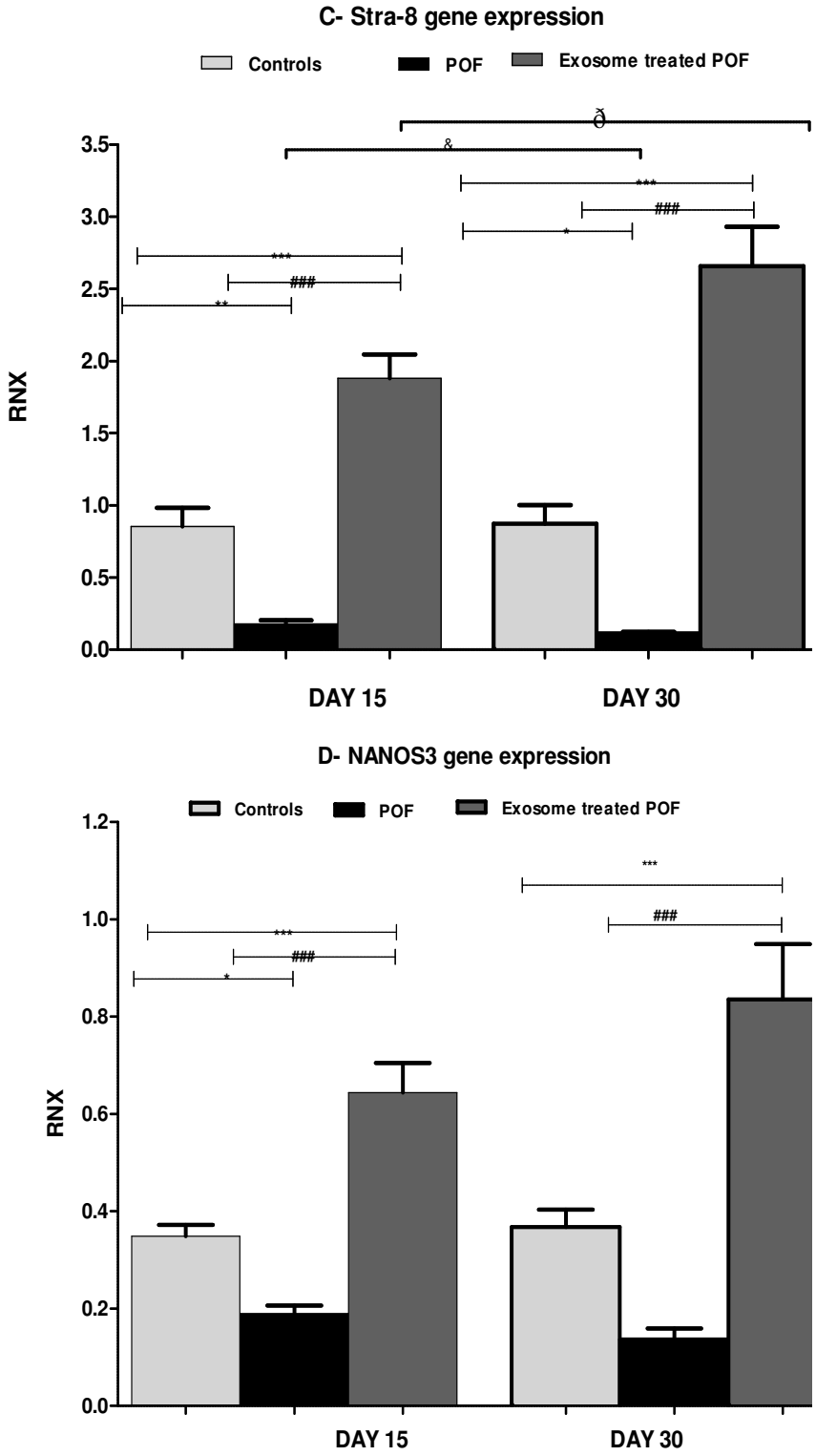

Figure 8b. Relative gene expressions of C) Stra8 and D) NANOS3 in rat study groups; Controls, induced premature ovarian failure (POF), POF treated with (Exosomes treated POF) 15 and 30 days after treatment. Data are expressed as RNX, relative expression (delta CT) where GAPDH was used as internal control gene. *Significantly different from respective controls ($P < 0.05$); **($P < 0.01$); ***($P < 0.001$). # Significant different from POF group (P value < 0.05); ##($P < 0.01$); ###($P < 0.001$), & and ð Significant different between 15 and 30 days of POF and exosomes treated repectively at $P < 0.05$.

Nanos3 genes which are required for normal oogenesis. The POF rat showed low gene expression of the four tested genes; (Figure 8a and b) compared to controls. P values were P <0.001 and P<0.01 for *Lin28,* P<0.05 and <0.01 for *Stra8* at 15 and 30 days respectively, P<0.05 for *Nanos3* at 30 days. As regard *Oct4*, it showed reduced expression but the P value was >0.05. This indicated suppression of; oocyte maturation, acquisition of mature follicles and reducing the germ cell pool considering the transcriptional, meiotic and survival process governed by their expression. The infusion of exosomes 7 days after cisplatin was associated with significantly higher expression of these genes compared to POF (P<0.001) at the two tested time points. Interestingly the expression of these genes were higher than the controls too P<0.001 at 15 and 30 days for *Oct4, Stra8, Nanos3,* and P values were <0.01 and <0.05 for *Lin28*. When comparing the expression levels of these genes at the tested time points of treatment *Oct4* and *Lin28* tend to be expressed at higher levels early after exosomes infusion (after 7 days after infusion), while *Stra8* and *Nanos3* were at later time point (21 days after infusion), indicating different temporal expression and probably different sources of these genes.

As regards to the ovarian morphological analysis, immunostaining, hormonal assay, gene expression of *Oct4, Stra8, Lin28* and *Nanos3*, the vehicle controls and POF groups (injected with DMSO only) showed no significant difference and were similar to their corresponding naïve controls and POF groups respectively at the tested 15 and 30 day time points (data were not shown). So, we opted to show the results comparing treatment groups to naïve controls and POF only.

DISCUSSION

To the best of our knowledge, this study is the first to demonstrate that infusion of BMSCs derived exosomes could restore ovarian function in a rat model of cisplatin induced ovarian failure.

Cisplatin induced ovarian failure is a common event in patients with cisplatin cancer chemotherapy (Nozaki et al., 2009). The gonadal toxicity is due to direct oocyte damage, apoptosis and necrosis of the granulosa cells of the primordial and primary follicles leading to its depletion, as well as atresia of the growing follicular population (Morgan et al., 2013). The molecular mechanisms underlying these gonadal toxicity is explained by cisplatin induced DNA damage via DNA double strand cross linkage, formation of cisplatin purine adducts leading to alteration of gene expression, activation of cell cycle check points which ends by cellular damage. In oocyte, the relatively resident cell, cisplatin was suggested to induce oxidative stress, and apoptosis via activation of both intrinsic and extrinsic pathways, uncoupling of the oxidative phosphorylation, and aberrant intra-cellular calcium homeostasis (Fernandes et al., 2016). The use of 2 mg/kg cisplatin presents a rat model of POF, in which depletion of oocyte, and apoptosis of the granulosa cell layers, an essential cellular component for maintenance of the oocytes were evident in ovarian sections at completion of induction of failure. Also terminal absence of the follicular population and corpora lutea with degeneration of the normal ovarian architecture, obliteration of ovarian vascularization, and ovarian fibrosis was also observed at the end of the experiment. The low levels of E2 and high levels of FSH were a reflection of these histological aberrations. These findings were reported previously by (Liu et al., 2014a).

As introduced earlier, the use of stem cell therapy for chemotherapy induced POF was investigated previously using many types of stem cells including; bone marrow, embryonic, amniotic fluid, adipose tissue, skin and menstrual blood derived stem cells. Some of these studies suggested direct differentiation of the injected stem cells into granulosa cells that subsequently provide hormonal support for oocyte maturation (Lai et al., 2013). Nevertheless, direct differentiation of stem cells into granulosa cell or oocyte was not accepted as the solitary restorative mechanism in other studies. Yet these cells were engrafted in the interstium or trapped in the thecal layers and induce certain paracrine effects within the oocyte microenvironment leading to reactivation of the host oogenesis (Lai et al., 2014; Liu et al., 2014a; Xiao et al., 2014). Consequently multiple molecular mechanisms have been suggested to mediate stem cell reparative effects on ovarian failure, including anti-apoptotic, anti-inflammatory, providing growth and trophic factors, and renewal of germline stem cells (Liu et al., 2012; Lai et al., 2015).

Extracellular vesicles released by stem cells are membranous structures loaded with bioactive molecules that mediate cell to cell communication. The current study showed that, the use of BMSCs derived exosomes treatment successfully restored the hormonal profile in the form of higher E2 and lowered FSH levels and rejuvenate the ovarian histological architecture compared to those untreated POF. This takes place through promoting de novo folliculogenesis and/ by inhibiting follicular atresia. The study further examined alterations in the expression of genes involved in regulation of apoptosis, meiosis, oogenesis, and maintaining germ cells to elucidate the exact reparative pathways.

Apoptosis of both oocyte and granulosa cells are implicated in the ovarian follicular atresia and luteolysis according to the follicular stage. *Survivin* is an anti-apoptotic molecule at caspase-dependent and independent levels. It also regulates mitosis by associating with mitotic spindle microtubules (Cheung et

al., 2011). *Survivin* mRNA was detected in oocytes and suggested to play an important role in oogenesis (Varras et al., 2012). Jiang et al. (2014) demonstrated that higher expression levels of *survivin* in women with tubal infertility than normal women protected the ovaries from follicular apoptosis. Moreover, disruption of *survivin* expression in oocytes and granulosa cells led to reduced ovulation and defective follicular growth, increased follicular atresia and impaired luteinization. According to the histopathological findings in this study lower *survivin* immunostaining in cisplatin treated rat ovaries was associated with follicular apoptosis and atersia while exosomes treatment showed enhanced *survivin* immunostaining that protects the granulosa cells from apoptosis and induce its cell division. Conservation of granulosa cells possibly preserved the gonadal somatic environment which surround oocyte and play a key role in folliculogenesis.

Consequently we proposed that exosomes treatment participated in restoration of early estrogen production and lowering the FSH through augmentation of the survival molecules like *survivin.* Tatone et al. (2006) reported that MSCs ameliorated chemotherapy-induced apoptosis of granulose cells in vitro by upregulating the anti-apoptotic markersBcl-2 protein. In the mean-time, *survivin* anti-apoptotic effect on the cumulus granulosa cells, which are in direct contact with the oocyte might also offered indirect positive control over the oocytes. Liu et al. (2012) used CD44+/CD105+ stem cells for transplantation treatment in premature ovarian failure. They found that, there was significant increase in the level of *survivin* after treatment.

Another possible mechanism for functional recovery of the ovary is the ability of exosomes to provide molecules that enhance meiotic division of oocyte and getting follicular maturation. *Stra8* (Stimulated by retinoic acid gene 8) is a cytoplasmic protein that is expressed in embryonic ovarian germ cells of vertebrates just before the 1st meiotic division (Krentz et al., 2011). *Stra8* is required for pre-meiotic DNA replication and the subsequent events of meiotic prophase, including chromosome condensation, cohesion, synapsis, and recombination. As sign-posted earlier cisplatin is known to attenuate the primordial germ cells line and induce metabolic changes and apoptosis of the oocyte. This was associated with marked reduction of *Sta8* expression in POF model. On the other hand, infusion of BMSCs derived exosomes attenuated the pathology and induced oogenesis through up-regulation of *Stra8* gene expression in the treated group. It has been reported that *Stra8* deficient mice were presented with small sized ovaries, and reduced number of oocytes (Anderson et al., 2008), while adipose tissue and amniotic fluid stem cell based therapy for POF also reported up-regulation of *Stra8* as an underlying mechanism for renewal of oogenesis (Fouad et al., 2016).

Oct4 (octamer-binding transcription factor) played an important role in the oogenesis both prenatal and postnatal (Monti and Redi, 2009). It is repressed by the start of meiotic prophase I then up-regulated at completion of the prophase I o f meiotic division indicating its specific role in oocyte growth and acquisition of meiotic competence (Kehler et al., 2004). In adult ovaries *Oct4* is also expressed at the time of growth phase of oocytes (Bahmanpour et al., 2013). It has been reported that the lower expression of *Oct4* results in apoptosis of the primordial germ cells and increase in antral and atritic follicles in adult ovaries (Johnson et al., 2004). This is in line with our model of POF in which lower *Oct4* expression at both mRNA and protein levels was associated with massive apoptosis and reduction of follicles of different growth stages. In the mean-time 2 weeks after exosomes therapy apoptosis was less and folliclugenesis was regained. Enhanced expression of *Oct4* on stem cell transplantation in POF model has been reported by Fouad et al. (2016).

Besides it protective role of the primordial follicles, and enhancing folliculogensis *Oct4* has been suggested to has an essential role in maintaining germ line stem cells and in inducing and establishing the pluripotency of many cell types. Interestingly, two types of germline stem cells derived from adult, even premenopausal ovaries possessed pluripotency as evidenced by the expression of *Oct4*; the oogonial stem cells (ovarian stem cells) and the surface epithelial stem cells (Lee et al., 2016). It has been also reported that overexpression of *Oct4* in these undifferentiated ovarian stem cells is liable to increase the efficiency of their differentiation into oocyte like cells (Lee et al., 2016). Although require further investigation this pathway is of great importance in cases of ovarian ablation with chemotherapy that is presented with massive follicular atresia and fibrosis. Even though, such types of ovaries are still capable of neo-oogensis/fulliculogensis according to Virant-Klun et al. (2011).

In the same way, *Lin28,* RNA-binding protein, is strongly related to pluripotency, cell differentiation and transition from pluripotency to a committed cell lineage (Hafner et al., 2013). *Lin28* is expressed in primordial and premeioticgerm cells co-localized with *Oct4* indicating its role in the conservation of the germline stem cell state via the regulation of microRNA activity in the developing ovary(Childs et al., 2012). Also, in mice ovary ablation of *Lin28* was associated with reduced PGCspool and reduced fertility (Shinoda et al., 2013). The current study demonstrated that *Lin28* showed the low level of expression in POF rat model applied in the current study however, levels were elevated on exosomes treatment. Noteworthy, the use of amniotic fluid stem cells and human induced pluripotent stem cells expressing *Oct4* and *Lin28* reported to restore POF by directly

differentiating into granulosa like cells (Xiao et al., 2014; Liu et al., 2016).

The current results showed that exosomes infusion up-regulated oocyte-specific transcriptional factor *Nanos3,* compared to rat ovaries that had been damaged by cisplatin. These data are in line with Lai et al. (2014) who reported nearly the same pattern of *Nanos3*expression in cyclophosphamide mice model of POF that was treated with skin derived MSCs.In addition Disruption of *Nanos3* in mice resulted in failure of survival and proliferation of primordial germ cell leading to infertility (Yamaji et al., 2010). Julaton and Reijo Pera (2011) reported that *Nanos3* expression human germ cell was highest in nuclei where the protein co-localized with chromosomal DNA during mitosis/meiosis and that reduced expression of *Nanos3* result in a reduction in germ cell numbers and decreased expression of intrinsic genes required for the maintenance of pluripotency and meiotic initiation and progression (Julaton and Reijo Pera, 2011).

This investigation indicated that exosomes infusion alleviated the cisplatin induced POF in rat in a similar way to what was achieved by stem cell based therapy, illustrating the potential role of exosomes as a novel therapeutic agent for treatment of POF.We suggested an array of mediators participated in modulation of ovarian function; including *survivin* that rescued the follicles from apoptosis, *Oct4* and *Lin28* that maintained the germ stem cell and the proposed ovarian stem cells, and finally, *Stra8* and *Nanos3* which judged its fate towards meiosis and oocyte formation. The higher levels of these genes in treated group compared to both POF and controls and the variations in the expression levels of these genes at different times *(Oct4* and *Lin28* showed higher expression at 15 days compared to 30 days after infusion of the exosomes the opposite were true for *Stra8* and *Nanaos3*) indicated different temporal expression and possibly different sources of these genes.

We suggested that exosomes infusion initially delivered and released its carriage of aformentioned effector proteins and RNA to the damaged ovaries inducing alterations in their micro-environment and modifying its biochemical and histological milieu towards normalization, that sequentially participated in the expression of these genes. Similarly,Xiao et al. (2016) showed that exosomes derived from amniotic fluid stem cells preserves ovarian follicles after chemotherapy. The authors suggested that miR-10a RNA carriage of the exosomes is the primary mediator for such effects. Similarly, the study byda Silveira et al. (2014) indicated that exosomes isolated from follicular fluid can regulate members of the TGFB/BMP signaling pathway in granulosa cells, and possibly play a role in regulating follicle maturation. Moreover, Santonocito et al. (2014) referred to a series of exosomal micro RNA that are highly represented in human follicular fluid and are involved in follicular maturation.

CONCLUSION

This study demonstrate for the first time that BMSCs derived exosomes infusion in POF rat model showed structural and functional reparative properties, possibly through delivering its carriage of protein, molecular contents and RNA bioactive molecules to the host ovarian niche inducing neo-oogenesis/folliculogenesis. This provides an effective and novel method for treating POF.

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