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Original Article

Ovarian surface epithelium mesenchymal stem cell ameliorates ovarian failure in chemically induced mice

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Abstract: Objectives: Primary Ovarian Failure (POF) is a primary ovarian failure characterized by amenorrhea or a premature loss of ovarian follicles before the age of 40. POF is characterized by low levels of estrogen hormone that increases the long-term risk for anxiety, heart disease, and osteoporosis. POF is, therefore, a major health concern because of impacting fertility and both cardiovascular and bone health. Yet there is no current cure for POF. Mesenchymal stem cells (MSCs) hold therapeutic potential for the treatment of POF. The aim of this study was to investigate the effect of MSCs derived from ovarian surface epithelium cells (OSE) on ovarian failure in C57BL/6 mice induced by cyclophosphamide (CTX). Methods: Seventy C57BL/6 adult female mice were divided into four groups: 25 non-CTX injected mice (a negative control injected with saline solution-Group I), and 25 CTX injected mice causing ovarian failure (Group II). Some CTX mice were injected with 0.5 ml of DMEM culture media into the tail vein (Group III). Another group of CTX injected mice were further injected with 0.5 ml of culture media containing 1 × 10^6 MSC into the tail vein (Group IV). Samples were used at 2 and 4 weeks for biochemical and histological analyses. Results: Group II and Group III showed a significant decrease in serum estrogen (E2) and Follicle Stimulating Hormone (FSH), with marked increase in progesterone levels. Administration of MSC to CTX mice significantly improved female reproductive hormones disturbances. Histological examinations showed increased follicle numbers and an apparent normal structure of ovarian follicles in the MSC group. Conclusions: This study suggests improved ovarianre generation and function and folliculogenesis after injection with MSCs derived from OSE, providing a conceptual framework for future mechanistic and translational studies in this area.

Keywords: Primary ovarian failure, ovarian surface epithelium stem cell, mesenchymal stem cells

Introduction

Primary Ovarian Failure (POF) is the primary ovarian failure or primary ovarian deficiency with absence of menarche (primary amenorrhea) or premature ovarian follicle depletion (secondary amenorrhea) at 40 years of age [1, 2]. It is estimated that about 1% of women younger than 40 and 0.1% under 30 are affected [3, 4]. Many individuals around the world are affected by infertility; studies have concentrated on discovering new therapies to treat and avoid infertility and improve the quality of life of infertile women and their families [5].

Women with POF experience menopausal symptoms, such as hot flushes, night sweats and vaginal dryness, similar to those going through a natural menopause [6]. Associated with the development of POF is the loss of fertility, which in most cases is due to the absence of follicles, and in other cases, the inability of remaining follicles to respond to stimulation [7]. In addition, there is increased risk of developing osteoporosis because of the lengthened time of exposure to reduced oestrogen. Treatment is linked to dealing with the menopausal symptoms, reducing the risk of osteoporosis and dealing with the loss of fertility.

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Like many disorders affecting our health and wellbeing, there is often an underlying genetic basis that may cause the disorder to occur in some individuals, or may simply predispose the individual to develop the disorder during their lifetime [8]. Given that POF does occur within family settings, it is clear that some gene defects may cause, or more likely be associated with, the development of POF. As there are few known causes of POF, it is possible that increased understanding of the molecular basis will arise from a greater understanding of the genes that may be implicated in the development of POF, pointing us in the direction of molecular pathways that may be defective. It is likely that this information may help us to diagnose the condition earlier, and therefore provide a way to save or protect remaining good follicles before the development of POF. It is also possible that if a better understanding of the biology of POF is achieved, this may lead to possible therapeutic avenues for defective follicles to be developed further.

Mesenchymal stem cells (MSCs) can differentiate into various cell types and regulate immune responses. Due to their immunomodulation roles, MSCs migrate to the site of damaged tissue and divide into cell types in this tissue under the induction of certain factors to repair the local microenvironment. Bone marrow, fat tissue, amniotic fluid, umbilical cord tissue, salivary glands, and dental pulp are all sources of MSCs [9-11]. Since MSCs are easy to isolate and amplify, they are widely involved in tissue damage repair by enhancing the function of endogenous cells and regulating the immune response [12].

MSCs are rich in bioactive factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and hepatocyte growth factor (HGF), which are useful for anti-apoptosis and anti-fibrosis that enhance premature ovarian failure (POF) [13, 14]. They can preserve ovarian functions, probably by inhibiting the inflammatory response and decreasing the oxidative stress. They educate immune cells to control the inflammatory response [15]. MSCs have a therapeutic potential for primary ovarian failure (POF) because they play a significant role in the restoration of POF induced by cisplatin in rats [16] or by chemotherapy in the mouse model. Notably, Bone marrow MSCS (BMSCs) can partially reverse infertility in chemotherapy-induced ovarian failure since BMSC-treated mice can develop ovarian hormones and reactivate folliculogenesis [17].

Ovarian Surface Epithelium (OSE) is a tissue that plays a role in the repair and regeneration of internal female reproductive organ tissue [18-22]. The OSE is separated from the basement membrane of the ovarian stroma and, underneath, by a dense collagenous connective tissue layer, the tunica albuginea, which is responsible for the whitish color of the ovary [18, 23]. Multiplegrowth factors, cytokines and proteins are expressed in OSE cells, including dermal protein, essential formative cell protein (bFGF), remodeling protein b (TGF-b), lamina, human sac endocrine (hCG), lymphokine (IL)-1/-6, several integrins, cadherins, and murine antigens such as mucin 1, cell surface associated (MUC1) and dehydrogenase 17-hydroxysteroid. These cytokines make internal female reproductive organ tissue easier to repair and regenerate [18-22].

Several studies have shown that the in vitro and in vivo ovarian follicle formation and differentiation are enhanced by ectopic expression of the transcription factor Oct4 in MSCs. Oct4 is known as a germ cell marker that plays a very important role during early folliculogenesis [24].

The goal of this study was to investigate the impact of MSCs derived from ovarian surface epithelium (OSE) cells on the ovarian failure induced by cyclophosphamide in C57BL/6 mice.

Materials and methods

Chemicals and media

All chemicals and media were procured from Sigma (St. Louis, MO, USA) and Gibco (Life Technologies, Burlington, ON, Canada), respectively, unless stated otherwise. Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 1 mg/ml polyvinyl alcohol (PVA) and 1% antibiotic-antimycotic solution (containing 10,000 IU and 10,000 µg/ml of penicillin-streptomycin, respectively) was used as washing media.

Ovarian surface epithelial (OSE) cell isolation

Internal female reproductive organ cells were collected according to assessment of ovarian

reserve (serum Estradiol E2), FSH and Progesterone and histology).

Mice were sacrificed under anesthesia induced with pentobarbital sodium injection (50-90 mg/kg body weight IP). After sacrificing of all mice, the specimen was placed into a sterile polypropylene container with approximately 25 ml of CO_2-independent culture medium and was transported to the laboratory at ambient temperature. The bloody specimen was rinsed with Hanks balanced salt solution (HBSS) or serum free culture medium. The rinse was kept and was cultured because it often contains OSE fragments. The specimen was held with forceps; surface down, over a 35-mm culture dish containing 2 ml of standard basal medium and the ovarian surface was scraped firmly with a white rubber scraper attached to a glass rod, or gently with a blunt instrument (sharp edges or excess pressure increase the risk of stromal cell contamination). The OSE fragments generated from scraping was incubated in the same culture dish where they were collected. Microscopically, they were appeared as small pieces of flat or rolled-up epithelial monolayers. Cells in the medium used for transport from surgery were collected by centrifugation for 5 min at 80 g and were plated into a separate 35-mm dish. All cultures were incubated at 37°C in humidified 5% CO_2/air and left undisturbed for at least 4 days. Culture medium with antibodies was changed as needed until the monolayers become confluent [25].

Culture of cells from the OSE

OSE fragments were attached to culture flask within a few days. Cultures should not be disturbed for the first 4 days. Medium was changed once a week while the cells were sparse, twice a week or more when they approach confluence. Confluent culture was rinsed once with Hanks' calcium, magnesium-free BSS with trypsin/EDTA (at room temperature). Fresh Hanks/trypsin/EDTA was added for 2-3 min (1.0 ml per 35-mm dish). Cell suspension was transferred to a centrifuge tube containing 2 ml of medium with 15% FBS to neutralize the trypsin. Then, was mixed and collected the cells by centrifugation at 1000×g for 5min at 24°C.

Fibroblast-like unit assay of colony formation

Fibroblast like colony growth was evaluated on primary cells grown in tissue culture six-well dishes. Total cells were plated at a density of 25×10^6 cells/well. After 7 days, the capability of OSE to form fibroblast like colonies was assessed. Images that showed OSE morphology were acquired by a contrast-phase microscope.

Viability test

The viability of stem cells was checked by Trypan blue exclusion according to the method of [26]. Trypan blue solution 0.4% (Sigma, USA) solution was diluted in saline 0.9% to yield 0.04% solution when mixed with the cells. Equal volume of both 0.04% trypan blue and stem cells were mixed and incubated for 10 minutes at 37°C. The number of viable cells (unstained) was counted using heamocytometer by light microscope. The number of viable stem cells/ml was then calculated according to the following equation.

$$\text{Viable cells\%} = \frac{\text{Number of viable cells/ml}}{\text{Total number of cells/ml}} \times 100$$

Expressions of cell surface antigen by flowcytometry

Ovarian surface epithelium (OSE) derived stem cells were detached via trypsinization for immunophenotypic analysis. Flow cytometry was performed using a BD Accuri™ C6 flow cytometer equipped with Accuri C6 software (version 1.0.264.21; Becton Dickinson, Franklin Lakes, NJ, USA). For staining, cells (1×10^6 cells/mL) were centrifuged at 1,200 rpm for 5 min and resuspended in phosphate-buffered saline (PBS). Cell suspensions (100 µL) were incubated with 10 µL of fluorochrome-conjugated monoclonal antibodies (mAbs; eBioscience, San Diego, CA, USA) for 30 min at room temperature in the dark. The antibody panel included antibodies targeting mouse antigens conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), and phycoerythrin-cyanine 5 (PE-Cy5), specifically: CD14 (Cat.No. biolegend-(50-164-085), FITC), CD44 (Cat.No.BD-pharmingen (553133), FITC), CD49 (Cat. No.Sony (1118525), PE), Oct4 (Cat.No.VWR (PRS176-327)), CD45 (Cat.No.BD-pharmingen (561874), FITC), and CD34 (Cat.No.NOVUS-biotechne-brand (NB600-1071), PE-Cy5). Following incubation, cells were washed twice, pelleted, and resuspended in PBS for acquisition. Cells were analyzed by setting a primary

gate on the healthy cell population based on forward scatter (FSC) and side scatter (SSC) profiles to exclude debris and doublets.

Experimental animals

Seventy (70) female C57BL/6 mice, aged 6 weeks and weighing about 20-23 g obtained from the animal facility of the Medical Experimental Research Center (MERC), Faculty of Medicine, Mansoura University, Egypt, were used in the present investigation. The animals were housed in separate metal cages (5 per cage) and allowed to become acclimatized to laboratory conditions for one week before the experiment. Fresh and clean drinking water was supplied ad-labium through specific nipples. Mice were kept in constant environmental and nutritional conditions throughout the period of the experiment. All experimental procedures were approved and supervised by the Institutional Ethical Committee for the care and use of laboratory animals in the Faculty of Science, Mansoura University, Egypt. All efforts were made to minimize animal suffering.

Induction of mous model with cyclophosphamide

Twenty-five mice at 6 weeks of age were used in this part of the study. To prepare recipients, wild-type females received 120 mg/kg of cyclophosphamide (Cytoxan; Bristol-Meyers Squibb, New York, USA), which induced damage to the primordial follicle cohort in the ovary. The ovaries were obtained under pentobarbital anesthesia and examined at 24 hr, 72 hr, 96 hr, 2 weeks, and 4 weeks to evaluate the chronic phase of ovarian damage and verify the self-healing process. This work was conducted in the theater of MERC.

Experimental design

The four groups of mice were divided as follows: 1. Group (I) (negative control, each contain 25 mice): Mice were injected intraperitoneally (i.p) at start point (Zero time) with a single dose of normal saline alone (with the same volume as in group II). 2. Group (II) (POF induction, each contain 25 mice): Mice were injected with a single dose of 120 mg/kg cyclophosphamide at start point (Zero time) (intraperitoneal (i.p) injection). 3. Group (III) (cyclophosphamide treated and DMEM culture media alone, each contains 10 mice): After 96 h premature ovarian failure (POF) disease induction. Mice were injected with 0.5 ml culture media into the tail vein. 4. Group (IV) (cyclophosphamide and stem cells treated, each contain 10 mice): After 96 h premature ovarian failure (POF) disease induction. Mice were injected with 0.5 ml of culture media containing 1×10^6 MSC into the tail vein.

Monitoring sex gland injury

Under physiological conditions, five mice were slaughtered with phentobarbital metal injection (50-90 mg/kg IP weight) at entirely different intervals, 24 hr, 72 hr, 96 hr, 360 hr, and 720 hr, in groups I and II. However, sexual glands and blood samples were taken at 2 and 4 weeks for groups III and IV.

Biochemical analysis

Five blood samples were obtained from all experimental groups during the biochemical analysis using an enzyme-linked immunosorbent assay kits during the above-mentioned period to estimate estradiolhormone (kit catalogue number LKE21 EURO/DPC, UK), gonadotrophic hormone (FSH-Catalogue number LKFS1 EURO/DPC, UK), and progestogen hormone level (catalogue number 1214583) from Roche company (Monobind, Inc., Lake Forest, CA, USA).

Histological analysis

Samples of ovarian tissue were taken from all the groups at intervals of 24 h, 72 h, 96 h, 360 h and 720 h (CI & CII) for microscopic analysis and at intervals of 2 and 4 weeks (CIII & CIV). Both tissues were stained with Hematoxylin and Eosin (H&E) stain on the four groups [23]. The tissues of the sex gland were ready for light-weight research [24]. The fixative was used in 100% solution of 0.34 N glacial ethanoic acid, 28% ethyl alcohol, the tissues were dehydrated by serial ascending alcohol concentration (BDH, UK) after fixation, and dissolving agent (BDH, UK), then the tissues were embedded in paraffin (Sheruood, USA). The paraffin-embedding tissue block was cut by scientific instruments (West Germany). The 4 μm sectional slide was stained with H&E and resorcinolphthalein.

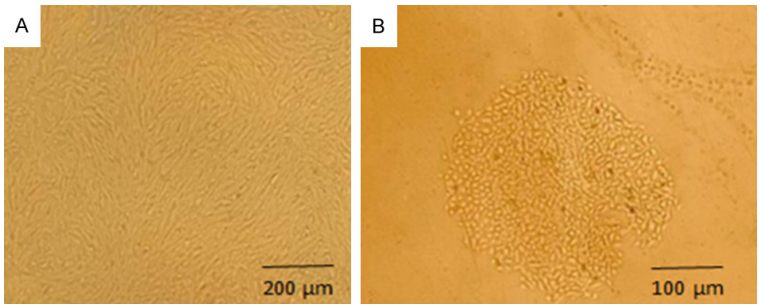


Figure 1. A. Ovarian surface epithelium stem cells under an inverted microscope at passage 3 were characterized by their fibroblast-like appearance (Original magnification ×200). B. Photomicrographs of OSE that grew in colonies and contained heterogeneous tiny pointed embryonic stem cells (Original magnification ×100).

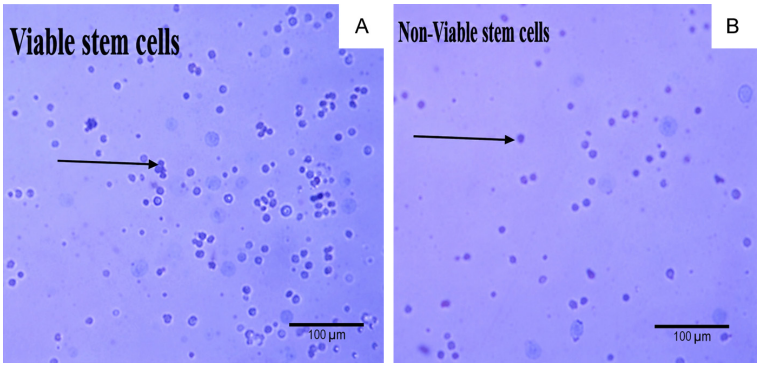


Figure 2. A. Light photograph of viable Mice OSE Stem Cells (↑). Mice ovarian MSCs didn't accept trypan blue stain. B. Light photograph of non-viable Mice OSE Stem Cells (↑). Mice ovarian MSCs accepted trypan blue stain. Scale bar is shown on each panel (Original magnification ×100).

Morphological evaluation of primordial and other follicle loss

The number of non-atretic primordial, primary and preantral follicles were then determined. Immature follicles were scored as atretic if the oocyte was degenerating (convoluted, condensed) or fragmented. Grossly atretic immature follicles lacking oocyte remnants were included in the analysis.

Statistical analysis

Statistical analysis of data was carried out by SPSS software program version 17. Results were expressed as mean ± standard deviation. Group differences were tested using paired sample student's t test and one way ANOVA followed by Tukey's Honest Significant Difference (HSD) post-hoc test for multiple comparisons. A p-value <0.05 was considered statistically significant.

Results

Harvest of ovarian surface epithelium (OSE) stem cells

Isolated OSE stem cells were characterized by their adhesiveness and fusiform, and star or spindle shapes as observed by an inverted microscope (**Figure 1A**). In addition, OSE stem cells were characterized by their adherence to plastic and growing as heterogeneous cell populations with predominant spindle-shape and forming cell like colonies (**Figure 1B**).

Viability of mice OSE stem cells

When isolated MSCs stem cells from the ovarian surface epithelial tissue were stained with trypan blue stain (Sigma, USA), viable stem cells didn't accept trypan blue stain, while non-viable cells accepted trypan blue stain (**Figure 2**).

Expression of the cell surface by flowcytometry analysis

MSCs showed positive expression for surface markers CD14 (91.32 ± 1.67), CD49 (91.97 ± 3.30), CD44 (92.7 ± 1.45) and Oct4 (94.6 ± 1.70) (Passage 3) and negative expression for surface markers CD45 (6.1 ± 2.11) and CD34 (7 ± 0.42) as presented in (**Figure 3**).

Induction of mous model with cyclophosphamide

CTX had successfully induced ovarian failure before injecting MSCs. That was confirmed by assessment of Samples at 24 h, 72 h, 96 h, 2 weeks and 4 weeks for biochemical and histological analyses. Group II, showed a significant decrease in serum estrogen (E2) and FSH, with marked increase in progesterone levels when compared to its basal level in mice group I. Moreover, investigation of tissue samples at the same times 24 h, 72 h, 96 h, 2 weeks and 4 weeks illustrated that marked increased in the number of atretic follicles, primary follicles,

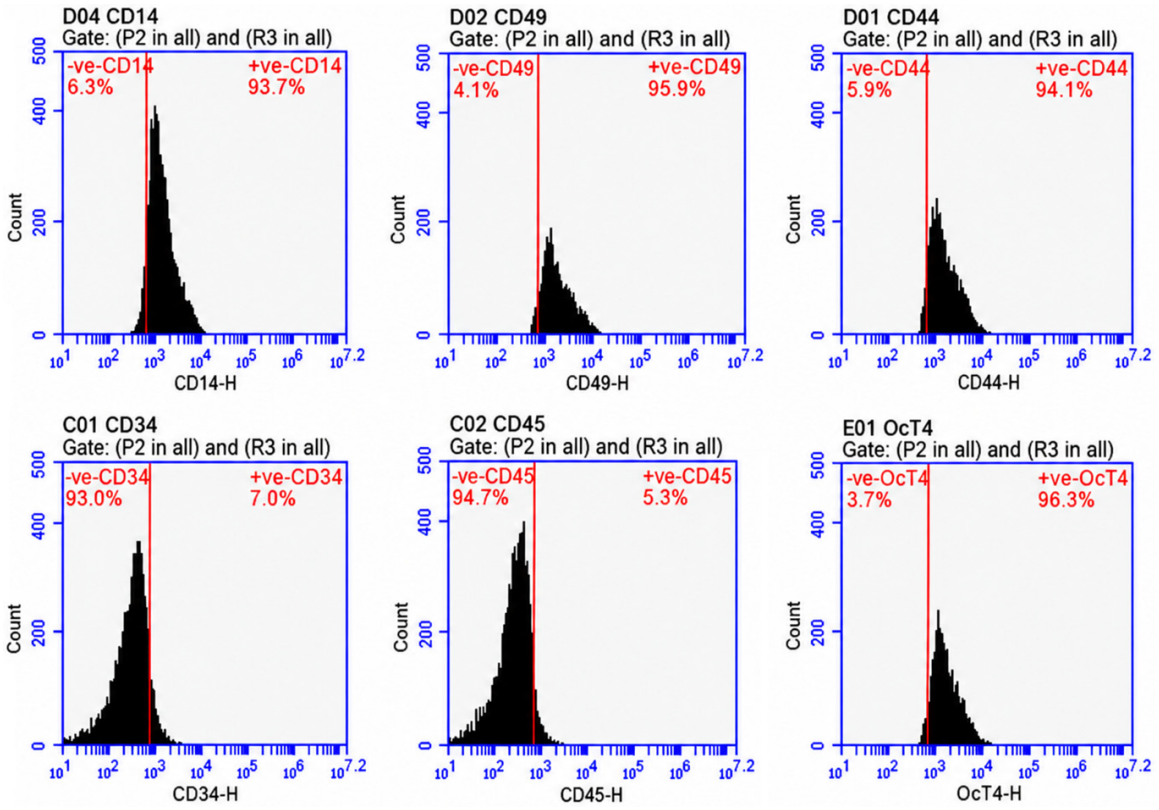


Figure 3. Phenotypic characteristic of mice ovarian surface epithelial mesenchymal stem cells, flow cytometry analysis revealed that their expression of surface antigens CD14, CD49, CD44 and Oct4 (Passage 3) was strongly positive and CD45 and CD34 was negative.

secondary follicle and primordial follicles compared to its basal level in mice group I.

Hormone levels of serum estradiol (E2), FSH and progesterone

The alterations in the internal female reproductive organ in group II mice was associated with a decrease of the mean body level of fluid estradiol (E2) after 24 hr of the last CTX injection, compared to its basal level in mice group I (31.5 ± 2.1 vs. 69.1 ± 3.4 ng/L, P<0.001). This decrease continued until 4th week in group II. Similar decrease was observed after culture media treatment in group III (19.7 ± 1.11 and 18.8 ± 1.16 vs. 69.3 ± 3.1 ng/L, P<0.001). Notably, there was no significance change in the amount of E2 in group IV mice that were injected with the culture media containing MSCs, compared to control group I mice (64.9 ± 3.17 vs. 69.3 ± 3.1 ng/L, *P*>0.05) in 4th week (**Figure 4A**).

The mean gonadotropic hormone level (FSH) was 0.174 ± 0.011 mIU/mL at 24 hr after the last CTX and still decreased in the 4th week in group II and group III mice, respectively compared to its basal level in mice group I (0.174 ± 0.011 and 0.168 ± 0.019 vs. 0.57 ± 0.012 mIU/mL, P<0.001). In group IV mice injected with MSCs at the 4th week, the concentration of FSH hormon increased to normal level when compared with the basal level in group I control mice (0.500 ± 0.053 vs. 0.57 ± 0.012 mIU/mL, *P*>0.05). The mean gonadotropic hormone levels also increased in group IV mice injected with MSCs from 2nd week till 4th week, compared to mice groups II and III (**Figure 4B**).

The internal female reproductive organ failure (group II) showed an increased of the mean body fluid progesterone level at 24 hr after the last CTX injection, compared to its basal level management in group I mice (0.766 ± 0.011 vs. 0.0334 ± 0.002 ng/L, P<0.001). Progesterone

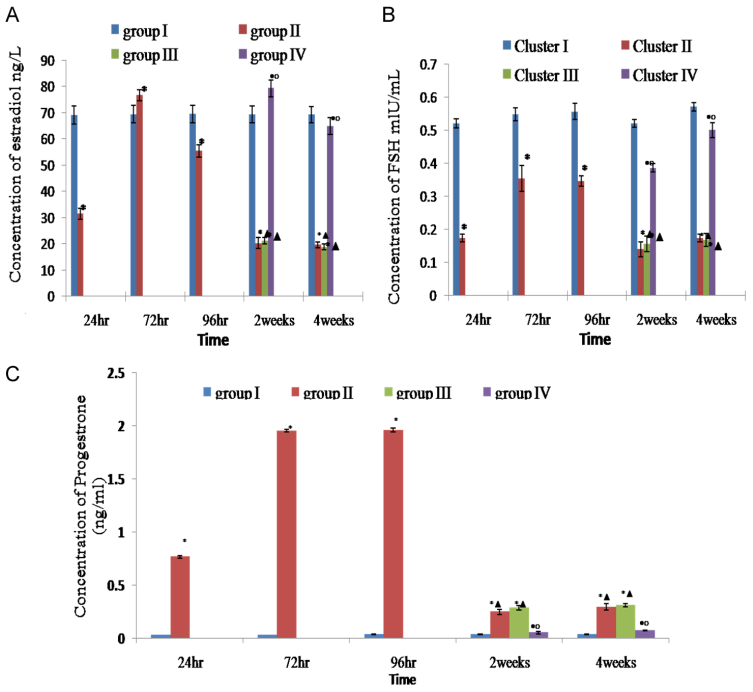


Figure 4. Hormonal analysis of the control and other experimental groups. (A) Estrogen (ng/ml), (B) Gonadotropic (FSH) hormone (mIU/mL), and (C) Progestogen (ng/mL) in the management fluid of the body and (POF) of mice, at 24 hr, 72 hr, 96 hr, 2 weeks and 4 weeks. * Significant difference (P<0.05) versus control I, ● versus II, ○ versus III, ▲ versus IV, Data are expressed as mean ± SD.

level continued to increased till the 4th week in group II and group III mice (0.294 ± 0.031 and 0.310 ± 0.016 vs. 0.0342 ± 0.002 ng/L, P<0.001). However, the level of progesterone was not significant in the 4th week in MSC-injected mice (group IV), compared to the amount of progesterone management in group I mice (0.0714 ± 0.003 vs. 0.0342 ± 0.002 ng/L, *P*>0.05). Interestingly, the mean progestogen levels showed decrease in MSC-injected mice (group IV) from the 2nd week till the 4th week, compared with other groups mice (II and III) (**Figure 4C**).

Histological analysis

Histological evaluations can help with determining the impact of MSC treatment on ovarian regeneration. Our Hematoxylin and Eosin (H&E)-stained sections (**Figure 5A-D**) showed that experimental studied groups of mice have aboriginal (primary, secondary and atretic). However, there's a big amendment within the degree of every aboriginal (primary, secondary and atretic), in the quantitative analysis described in section 3.6, and a comparison between all groups were ascertained in **Figure 6** for every parameter.

Number of follicles

As shown in **Figure 6A**, the number of atretic follicles in ovarian failure (group II mice) showed increased in number along the times 24 hr, 72 hr, 96 hr, 2 weeks and 4 weeks after injection, compared to group I control mice (P<0.001). The number of atretic follicles/HPF increased in mice treated with cyclophosphamide and medium (group III) at 2 and 4 weeks, compared to group I control mice (P<0.001). The number of atretic follicles/HPF was not significantly changed in group IV mice at 2 and 4 weeks, compared to control group I mice (P>0.05 and >0.05). The number of atretic follicles/HPF decreased in group IV mice, compared to ovarian failure in group II mice at 2 and 4 weeks (P<0.001).

Notably, **Figure 6B** showed that the number of primary follicles/HPF redoubled in the POF ovary in group II after CTX injection at 24 hr, 72 hr, 96 hr, 2 weeks and 4 weeks, compared to group I control mice (P<0.001). The number of primary follicles/HPF decreased in MSCs in group IV compared to the ovarian failure in group II (P<0.001). In addition, the number of primary follicles/HPF in MSCs in group IV showed insignificant difference at 2 and 4 weeks compared to group I (P>0.05 and >0.05; **Figure 6B**). The number of primary follicles/HPF in MSCs in group IV was greatly decreased at 2 and 4 weeks compared with group III (P<0.001; **Figure 6B**).

Figure 6C showed that the number of secondary follicles/HPF in the POF ovary increased at 24 hr, 72 hr, 96 hr, 2 weeks and 4 weeks in group II compared to group I (P<0.001). In addition, the number of secondary follicles/HPF increased in group III at 2 and 4 weeks com-

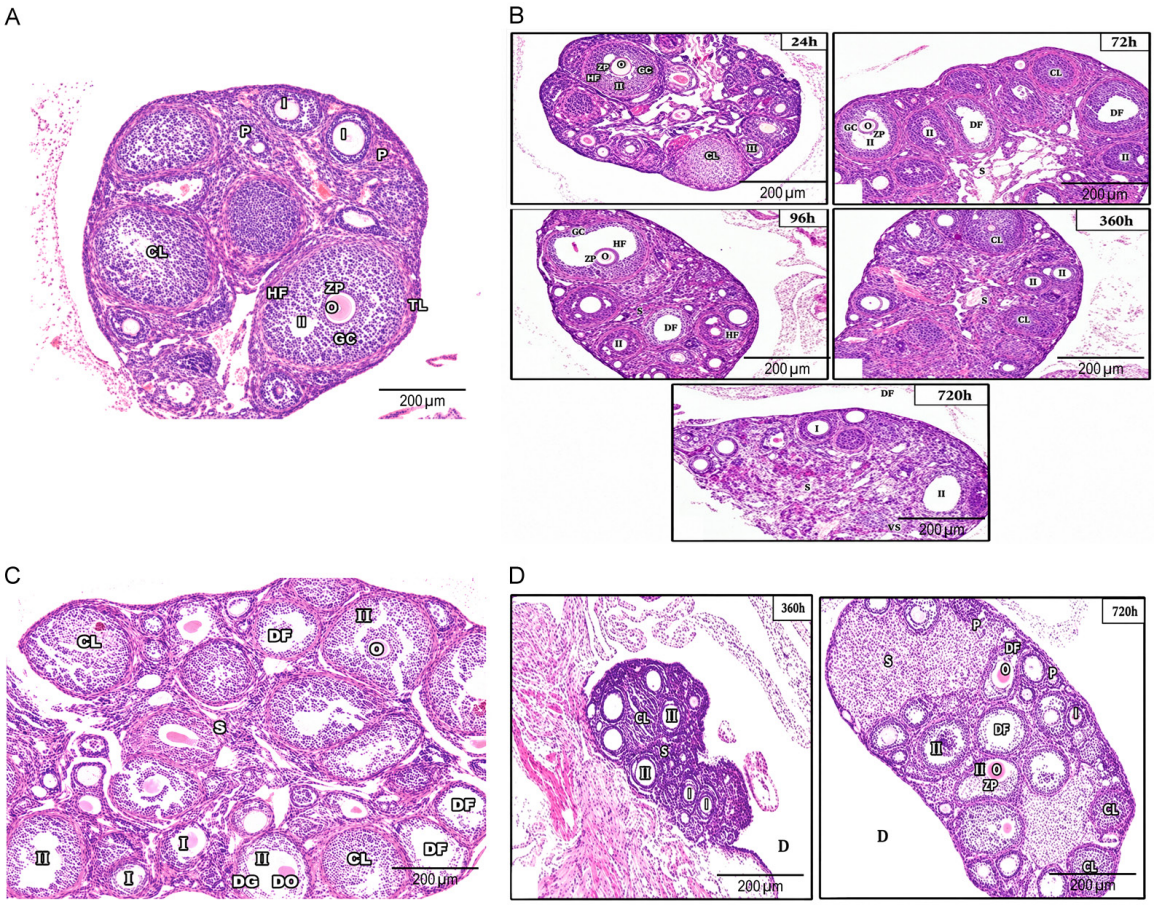


Figure 5. Hematoxylin and Eosin (H&E)-stained ovaries showed normal ovary in Group (I) control mice. Scale bar = 200 µm (H&E, 100×) (A). H&E-stained ovaries CTX failure ovary showing decreased sex gland mass in Group II. Bars of Scale = 200 µm (H&E, 100×) (B). Histopathological examination of mice ovary in Group III. Note decreased buxom demarcated outer cortex (Ct) and inner medulla (Md). Bars of Scale = 200 µm (H&E, 100×) (C). Histopathological examination of mice ovary in Group IV. Note more formation of sex glands and blood vessel follicles are shown by the recipient group of MSCs at 2 weeks and 4 weeks after injection of MSC into the tail vein. Bars of Scale = 200 µm (H&E, 100×) (D). Abbreviations: Germ Cells, HF: Healthy Follicle, CL: Corpus Luiteum, DF: Damage Follicles, GC: Granulosa cells, I: Primary Follicles, O: Oocyte, II: Secondary Follicle, ZP: Zona Pelucida, P: Primordial, VS: Vacuolated Stroma, S: Stroma, DG: Damage Granulosa, DO: Damage Oocyte.

pared to group I (P<0.001). Moreover, the number of secondary follicles/HPF showed insignificant distinction in group III compared with ovarian failure in group II at 2 and 4 weeks (P>0.05). The number of secondary follicles/HPF also showed insignificant distinction in MSCs (group IV) at 2 and 4 weeks compared with group I (P>0.05 and >0.05). Moreover, the number of secondary follicles/HPF decreased in MSCs group IV at 2 and 4 weeks compared with ovarian failure in group II (P<0.001). The number of secondary follicles/HPF decreased in MSCs group IV at 2 and 4 weeks compared with group III (P<0.001; **Figure 6C**).

Finally, **Figure 6D** showed that the number of primordial follicles/HPF increased at 24 hr, 72 hr, 96 hr, 2 weeks and 4 weeks in group II compared to group I (P<0.001). In addition, the number of primordial follicles/HPF increased at 2 and 4 weeks in group III compared to group I (P<0.001). However, the number of primordial follicles/HPF showed insignificant distinction at 2 and 4 weeks in group III compared with group II (P>0.05 and >0.05, **Figure 6D**). The number of primordial follicles/HPF also showed insignificant distinction at 2 and 4 weeks in MSCs (group IV) compared with group I (P>0.05 and >0.05). Notably, the number of primordial follicles/HPF decreased at 2 and 4 weeks in MSCs group IV compared to the ovarian failure group II (P<0.001). The number of primordial follicles/HPF decreased at 2

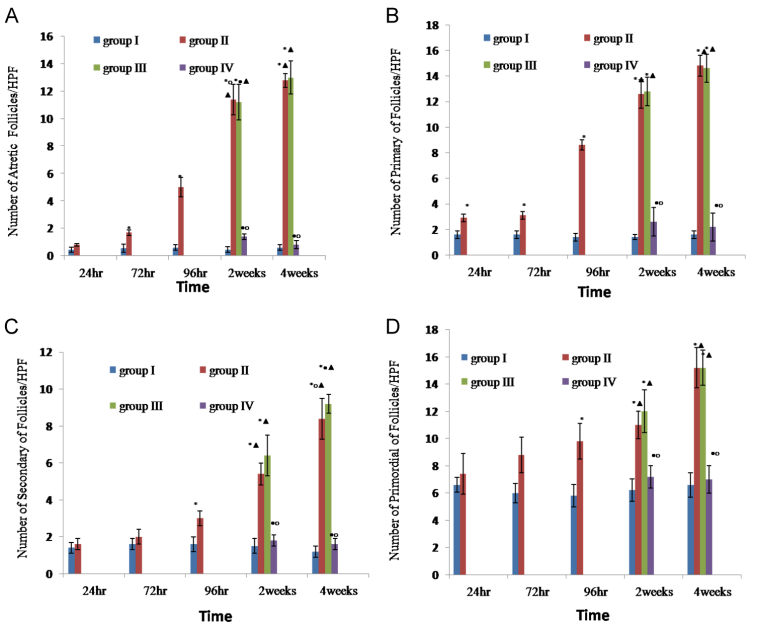


Figure 6. Ovarian histopathological analysis of the control and other experimental groups. A. Numbers of atretic follicles/HPF. B. Primary follicle/HPF. C. Variety of secondary follicles/HPF. D. Primordial of follicles/HPF in management ovaries and (POF) at 24 hr, 72 hr, 96 hr, 2 weeks and 4. * Significant difference ($P<0.05$) versus control I, ● versus II, ○ versus III, ▲ versus IV, Data are expressed as mean ± SD.

and 4 weeks in MSCs in group IV compared to group III ($P<0.001$; **Figure 6D**).

Discussion

The present study demonstrated that isolated MSCs from the OSE tissue consist of a heterogeneous population of cells with predominant spindle-shape morphology. They are ready to differentiate into colonies of formative, or colony-forming, cells that function to maintain and restore internal female reproductive organ tissues. These results agree with previous reports on the existence of precursor pluripotent stem cells among OSE cells in human ovaries. These reports also showed evidence that OSE cells have persuasive functions in the maintenance and repair of internal female reproductive organ tissues [18, 21].

We found that MSCs derived from epithelial surface tissue of mice gonads express the cell surface markers CD29, CD44 and transcription factor Oct4, in agreement with previous research showing high expression levels of CD29, CD44 and other cell surface markers, including CD90, CD73 and CD105 (>95%) in MSCs derived from epithelial surface tissue of mice gonads [27]. Our study also showed a negative expression of cell surface markers CD45, CD34 and a very low expression level of CD45 and CD34 (<2%), similar to previous reports [27]. Therefore, the conception of sex gland damage was not a correct definition of gestation. The changes in sex glands were established by chemical and microscopic anatomy findings, as shown in the current studies. Interestingly, Estrogen (E2), follicle-stimulating hormone (FSH), and progesterone levels were not assessed for the recovery of the sex glands and somatic cell action [28].

This study showed a remarkable decrease in both estrogen (E2) and FSH hormones, but an increased level of progesterone hormone in groups (II, III) mice after cyclophosphamide (CTX) injection, as explained in the results of the CTX on the primordial sex gland reserve cyst (source of E2). Notably, approximately half of the primordial sex glands destroyed after the initial intraperitoneal CTX injection [29]. Indeed, a remarkable decrease in antepartum follicle growth, but an increased follicle degeneration in murine ovaries were observed when using a daily dose of 120 mg CTX/kg body weight per day for 14 days [30].

Similar to previous studies, we detected notable narrowing and obliteration of sex gland blood vessels following CTX injection. These findings may explain systemic injury of the ovary, leading to cyst failure, and suggest a broader effect of CTX treatment that is not limited to the follicle only in the ovary [31].

Our results agree with previous findings that hypergonadotropinemia, hypoestrogenic and failed recovery of the ovary, in addition to increased E2, follicle-stimulating hormone but decreased progesterone in Group IV recipients are also demonstrated by sex gland failure in Group II and Group III culture media. Notably, we also did not observe major differences in the levels of E2, progestogen, and follicle-stimulating hormones between the fourth week of injection and the pace group. In addition, im-

proved sex glands, increased cyst numbers, gaining typical sex gland follicle structure, and corpora lutea antral follicles are observed in stem cell treated groups IV [32-35]. Interestingly, several studies showed that, even though BM-derived germ cells exhibit an organic process block, both premature sex gland failure and sterility determined in treatment-conditioned female mice are often saved by transplantation of BM from untreated female donors [36-39].

Interestingly, previous studies on Follitropin Receptor Knockout mice (FORKO) lacking the FSH receptor demonstrated that intravenously injected BM-derived MSCs can reach the ovaries and help restore their function, which can lead to normalized levels of FSH and steroid sex hormones and restoring folliculogenesis [40, 41].

Research on the human OSE stem cells show that they can develop a variety of cell types, including oocyte-loke cells in vitro. Notably, the human OSE stem cells can preserve many of embryonic stem cell characters. They may also produce multiple distinct cell types such as new eggs in vitro, particularly when derived from patients with premature ovarian failure or postmenopausal ovaries [42, 43].

Finally, since research studies show that human oocytes can be developed in vitro from OSE cells growing in culture, this method may be a potential treatment or fertility for women who have no or few follicles in their ovaries (e.g., women with premature ovarian failure) [27-43]. The main mechanism described in current research involves the differentiation OSE-derived stem cells and their subsequent maturation into oocytes, often using microinjection or transplantation techniques to provide an appropriate environment (in vivo or ex vivo) for this process to occur. In addition, MSCs secrete several bioactive variables that can influence sex gland regeneration and repair. For example, MSCs can secret VEGF growth factor that prevents cell death, enhances tissue properties and stimulates tissue regeneration. Jointly, VEGF can help interact with the somatic cell niche that supports the renewal method [42, 43].

Conclusion

In conclusion, our study suggests improved ovarian regeneration and function and folliculogenesis after injection with MSCs derived from OSE, providing a conceptual framework for future mechanistic and translational studies in this area.

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Disclosure of conflict of interest

None.

Abbreviations

bFGF, basic fibroblast growth factor; BMSCs, Bone marrow stem cells; CTX, cyclophosphamide; E2, estrogen; FORKO, Follitropin Receptor Knockout mice; FSH, follicle stimulating hormone; MUC1, mucin 1, cell surface associated; HBSS, Hanks balanced salt solution; H&E, Hematoxylin and Eosin; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; bFGF, essential formative cell protein; TGF-b, remodeling protein b; hCG, lamina, human sac endocrine; IL, Interleukin lymphokine (IL)-1/-6; MSCs, Mesenchymal stem cells; OSE, Ovarian Surface Epithelium; PBS, phosphate buffered saline; POF, Primary Ovarian Failure; TGF-β, Transforming growth factor-beta; VEGF, vascular endothelial growth factor.

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