Original Article

Role of Heat Shock Pretreated Bone Marrow Mesenchymal Stem Cells Versus Non-Treated Ones in Repair of Experimentally Induced Premature Ovarian Insufficiency by Doxorubicin

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ABSTRACT

Introduction: Premature ovarian insufficiency (POI) is one of the most severe problems threatening the reproductive health of women of normal childbearing age.

Aim of the Work: This study aimed to throw a light on the effect of heat shock (HS) pretreated bone marrow mesenchymal stem cells (BM- MSCs) versus non-treated ones in repair of DOX-induced POI.

Material and Methods: Fifty-six adult female albino rats were utilized and divided into three groups; Stem cells donor group included 10 rats, Control Group included 16 rats, Experimental group (30 rats); each rat received (1ml) 2.1 mg/kg BW Doxorubicin (DOX) once every 3rd day for seven IP injections. Then, this group was subdivided into three subgroups (n=10 each); subgroup III-A (DOX- treated), subgroup III-B (DOX+ routine BM-MSCs) and subgroup III-C (DOX+ HS-pre-treated BM-MSCs). Green fluorescent protein (GFP) was used for labeling and tracing the injected BM- MSCs. E2 and FSH levels were measured, rats were sacrificed and ovarian specimens were possessed for histological, immuno-histochemical, morphometric studies and statistical analysis.

Results: The ovarian function in rats that received heat shock pre-treated BM-MSCs revealed significant increase of the serum level of E2 and significant decrease of FSH than in rats received routine ones. Heat shock pre-treatment increased homing of BM-MSCs which was proved by the significant increase of fluorescence traced GFP-labeled BM-MSCs in subgroup III-C as compared to subgroup III-B. There was marked improvement in the histological structure of ovarian sections of the rats received heat shock pre-treated BM-MSCs more than those received routine ones with significant decrease of apoptosis evidenced by caspase-3 immunoreactivity.

Conclusion: Heat shock pre-treated BM-MSCs had a great effect in reducing the degenerative changes associated with DOX induced POI than the routine ones.

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Key Words: BM-MSCs, doxorubicin, GFP, heat shock, POI.

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INTRODUCTION

Degenerative alterations in the ovarian tissue lead to reduced ovarian reserve and insufficient ovarian sex hormones known as premature ovarian insufficiency (POI). This condition causes menopause to begin before the age of forty and a rapid decline in ovarian function[1]. POI has grown in prominence as a leading cause of infertility in reproductive-aged women; it has devastating effects on patients' emotional and physical well-being and places a heavier financial strain on families and communities[2]. POI may have many different causes, including hereditary, autoimmune, iatrogenic, or even anticancer treatment side effects[3].

Doxorubicin (DOX) is an anthracycline antibiotic anticancer drug that is proved to be effective against different types of cancers. It is one of the most commonly used to treat soft tissues and bone sarcomas and malignant tumors of breast, thyroid, ovary and bladder. It is also used to treat acute lymphoblastic leukemia[4]. It was reported that anthracycline chemotherapeutic drugs as DOX was associated with a risk of treatment-induced POI in 40–60% of the patients with breast cancer before the age of forty. DOX targets healthy tissues with high proliferating rates, such as gonads leading to gonadotoxicity[5,6].

The original source of mesenchymal stem cells was bone marrow. The umbilical cord and adipose tissue are two examples of the many tissues from which they may be extracted. Because of their unique properties including minimal immunogenicity, broad sources, availability, and immunomodulatory activity MSCs-mediated treatment is useful in a variety of illnesses[7,8].

Recently, regenerative medical research and animal experiments have proved that BM-MSCs transplantation can reverse chemotherapy induced ovarian damage only partially[9]. Using BM-MSCs for tissue regeneration has faced some impediments, including poor homing to the

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injured tissue and apoptosis after transplantation, so their transplantation did not attain the expected repairing effect[10,11].

Pretreatment of MSCs is a novel strategy of stem cell in *vitro* preconditioning to enhance their capacity to migrate, which improves therapeutic effects and regenerative capacities of the injected cells[12]. Heat shock (HS) is one of the pretreatment preconditioning methods of BM-MSCs that has been reported to increase their survival and enhance their function[13].

It may be difficult for MSC-based treatment techniques to enter clinical practice due to a lack of sufficient information on MSC migration and homing towards injured tissues. Therefore, stem cell tracking is essential for tracking their destiny and bio dispersion[14].

An in *vivo* cell-tracing strategy that may be used to monitor the distribution and vitality of implanted BM-MSCs in real time and without invasive procedures involves labeling of stem cells[15]. Since it is highly lipophilic, green fluorescent protein (GFP) labels all cell types quickly and effectively, making it one of the indirect labeling techniques. Following tissue collection, cells tagged with GFP may be studied using a fluorescence microscope[16].

Therefore, this study was designed to determine how heat shock pretreatment of BM-MSCs repaired doxorubicin-induced premature ovarian insufficiency in adult albino rats, this study compared the effects of untreated and treated cells. The histological and immunohistochemical analyses were carried out using fluorescence microscopy to trace the transplanted GFP-labeled BM-MSCs.

MATERIALS AND METHODS

Animals

This experimental study was done using fifty-six adult female Wistar albino rats aged 8 weeks with an average weight ranging from 150-200 gm /each. They were obtained from the animal houses, Faculty of Medicine, Cairo and Tanta Universities. They were kept in clean properly ventilated separate cages (five rats per cage) and received a balanced diet and free water supply two weeks before the experiment for acclimatization. Care of animals as well as all steps of the experiment were carried out according to the rules and regulations laid down by the research ethics committee of animal's experimentation, Faculty of Medicine, Tanta University (approval code 34442 /2/21).

Chemical

Doxorubicin (DOX); Adriamycin vials used in this study were obtained from Pfizer Pharmaceutical Industry, Cairo. Each vial containing DOX in a concentration of 50 mg/25ml was dissolved in 0.9 % normal saline solution. Each 1 ml DOX was dissolved to form a 7 ml solution of the drug. Each rat of the experimental group was given 1ml each injection containing 0.42 mg of DOX (2.1 mg/kg BW) to reach after 7 injections cumulative doses 3mg (15 mg/kg BW)[17].

Experimental Design

Vaginal smears were obtained daily from each rat and only that showing at least two consecutive normal 4-days vaginal estrus cycle was included in the experiment[18]. The rats were divided into three groups as follows:

Group I "Stem cells donor group": It consisted of 10 rats used as bone marrow donors from which BM-MSCs were isolated, cultured and labeled.

Group II "Control Group": It included 16 rats which were subdivided into three subgroups: Subgroup II-A (Normal control): consisted of 4 rats; received no medication

Subgroup II-B (DOX- vehicle control): consisted of 4 rats; each one received 1 ml 0.9 % normal saline by intraperitoneal injection (IP) once every 3rd day for 3 weeks and were sacrificed one week after injection[19]. Subgroup II-C (DOX +BM-MSCs vehicle control): consisted of 8 rats; each one received normal saline the same as subgroup II-A and one week later, 0.5 ml of phosphate-buffered saline (PBS) was injected into its tail vein. 4 rats were sacrificed after one week and the other 4 after three weeks after injection[20].

Group III "Experimental group": It included 30 rats; each rat received 1 ml DOX (2.1 mg/kg BW) once every 3rd day by seven intraperitoneal injections, in cumulative doses 15 mg/kg BW over a period of 3 weeks[17]. After confirmation of POI by hormonal assay (the mean E2 level was (32.77 ± 1.93) and the mean FSH level was (21.46 ± 1.37), this experimental group were subdivided into three subgroups each one consisted of 10 rats:

1. Subgroup III-A (DOX treated): rats were sacrificed one week after the last dose of DOX injections[19].
2. Subgroup III-B (DOX+ routine BM-MSCs): each rat received single dose of 3×106 routine BM-MSCs suspended in 0.5 ml phosphate buffered saline injected into its tail vein one week after the last dose of DOX injections[20,21].
3. Subgroup III-C (DOX+ HS-pre-treated BM-MSCs): each rat received single dose of 3×10^6 heat shock (HS)-pre-treated BM-MSCs suspended in 0.5 ml phosphate buffer saline injected into its tail vein one week after the last dose of DOX injections[22].

Rats of subgroups III-B and III-C were sacrificed 1 and 3 weeks after BM- MSCs injection (5 rats after 1 week and 5 rats after 3 weeks)[20,23].

Preparation of bone marrow-derived mesenchymal stem cells (BM-MSCs)

BM-MSCs were prepared at the Egyptian society for Progenitor Cell Research - Cairo.

Isolation and culture of BM- MSC[24,25]

Rats were administered pentobarbital sodium for anesthesia. Subsequently, both femurs and tibiae were

collected and flushed with a complete culture medium comprising 10% FBS. After centrifugation at a speed of 2500 rpm for a duration of 20 minutes, the cells were subjected to two washes with PBS. Subsequently, the cells were resuspended in DMEM complete medium supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin. The cells were then incubated at 37°C in a humidified environment with 5% CO_2 concentration. At the 48-hour mark, the initial medium change was carried out to eliminate non-adherent cells. Once the cells reached 90% confluence, they were harvested using 0.25% trypsin and subsequently passaged at a ratio of 1:3. Non-adherent cells were removed after 72 hours, following which the fresh culture medium was replenished

BM -MSCs in the 3rd passage (after two weeks) were divided into two parallel groups

A. The routine BM -MSCs subgroup was incubated under typical conditions (cells were then incubated at 37°C in a humidified atmosphere with 5% CO_2)

B. The heat shock (HS) pretreated BM-MSCs subgroup was incubated with HS pretreatment.

Heat shock (HS) pretreatment of BM-MSCs: The cells were exposed to heat shock under the optimal condition by placing the culture flasks inoculated with BM- MSCs into a 42 °C water bath for 1 hour. Then, they were inoculated at 37 °C in an incubator with 5% CO_2 for 48 hours[22,26].

Labeling of BM-MSCs with green fluorescent protein (GFP) was achieved using an in *vitro* approach. This involved the addition of a plasmid, specifically cytomegalovirus Aequorea coerulescens green fluorescent protein (pCMV-AcGFP), mixed with lipofectamine at a ratio of 2:1 to each plate. The plates were then incubated at 37°C for 6 hours prior to transplantation. During the incubation period, the flask containing the cells was gently shaken every 15 minutes for a total of 2 hours. Then, the culture medium, which consisted of 10% FBS, was added to the flask.BM-MSCs labeled with GFP were observed as distinct green fluorescent dots under a fluorescence microscope in the ovarian sections of subgroup III-B and subgroup III-C after injection[27,28].

Biochemical study

Blood samples were withdrawn from the retro-orbital venous plexus using capillary tubes into dried clean centrifuge tubes. The serum level of Estrogen (E2) and follicle- stimulating hormone (FSH) were assessed according to routine biochemical analysis system using clinical test kits (Biotechnology Company, Shanghai) by enzyme-linked immunosorbent assay (ELISA)[29-30].

Mortality rate recording

Mortality rate was recorded in the three groups and any deaths occurred throughout the experiment were replaced.

Histological and Immunohistochemical studies

At the end of the experiment, the rats were anesthetized and sacrificed. Both ovaries of each rat were extracted and fixed in 10% neutral buffered formalin then, ovarian specimens were processed to be stained by:

- Hematoxylin and eosin stain: to study the normal histological structure of the rat ovary and any histopathological changes among the various experimental groups.
- Caspase-3 stain for detection of apoptosis as follows[31]:

Sections were dewaxed and rehydrated then; they were incubated with ready-to-use primary rabbit polyclonal antibodies for caspase-3((CPP32 Ab-4, RB-1197-PCS, Lab Vision Corporation, United Kingdom), for 30 minutes. The slides were washed 3 times for three min with PBS. A secondary antibody was added to tissue sections and co-incubated for 30 min then washed 3 times for three min each with wash buffer, followed by counterstaining with Mayer hematoxylin before examination under the light microscope. Positive caspase-3 immunoreactivity appeared as brownish cytoplasmic discoloration of the cells while the negative ones remained blue. Positive control tissue (tonsil).

Morphometric

Study was performed using the Image J program analysis software for measuring the following parameters:

1. Green fluorescent protein (GFP) fluorescence intensity of BM-MSCs in ovarian tissues[32].
2. Counting the ovarian follicles (primordial, preantral, antral and atretic)[20,33] and calculating the percentage of healthy and atretic follicles from the following equations[34]:
    - The % of healthy follicles = {number of (primordial + pre-antral + antral) follicles / total number of follicles} × 100.
    - The % of atretic follicles = {number of atretic follicles / total number of follicles} × 100.
3. Semiquantitative analysis of caspase-3 immunoreactivity[35] and scoring of apoptotic cells' percentage (Immune score; from 0 to 4)[33].

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and analyzed by using Statistical Package for the Social Sciences(SPSS) program version 20 (simple t-test). A difference among the means of a $P\ value \leq 0.05$ was considered statistically significant and a $P\ value \leq 0.001$ was considered statistically highly significant. Whereas a $P\ value > 0.05$ was considered insignificant[36].

RESULTS

Mortality rate

Stem cells donor and control groups showed no deaths

throughout the experiment. However, in the Experimental group the mortality rate was observed (26.7%) and the dead rats were replaced.

Biochemical Study(E2 and FSH hormone levels)

Subgroup III-A (DOX- Treated) showed a highly significant decrease in the mean E2 level (32.77 ± 1.93) and a highly significant increase in the mean FSH level (21.46 ± 1.37) as compared to control subgroups with *P value* (0.001). In subgroup III-B (DOX+ routine BM-MSCs) there was significant decrease in the mean E2 level (57.22 ± 4.61) and significant increase in the mean FSH level (8.99 ± 1.10) as compared to control subgroups, this group also showed a highly significant difference in the mean serum level of E2 and FSH as compared to subgroup III-A with *P value* (0.001). Moreover, in subgroup III-C (DOX+ HS-pre-treated BM-MSCs), there was a highly significant difference in the mean serum level of E2 (61.45 ± 3.21) and FSH (7.64 ± 0.85) as compared to subgroup III-A with *P value* (0.001). Regarding subgroups IIIB and IIIC there was a significant difference in the mean serum level of E2 and FSH with *P value* (0.045 and 0.046 respectively) (Table 1).

Green fluorescent protein (GFP) labeling of BM-MSCs with its fluorescent intensity

In subgroup III-B (DOX+ routine BM-MSCs), the ovarian sections of the rats sacrificed after 1week revealed multiple GFP-labeled routine BM-MSCs located in the ovarian interstitial tissue and surrounding the follicles but absent in the ovarian follicles (Figure 1A). After 3 weeks, fewer GFP-labeled routine BM-MSCs were noticed in the ovarian section than after 1 week (Figure 1B). There was a highly significant decrease of the mean GFP fluorescence intensity of routine BM- MSCs between 1 and 3 weeks (58.32 ± 6.35 and 26.37 ± 4.18 respectively) .In subgroup III-C (DOX+ HS-pretreated BM-MSCs), the ovarian sections of the rats sacrificed after 1 week revealed numerous GFP-labeled HS- pretreated BM-MSCs more than that of subgroup III-B after the same period (Figure 1C) with significant increase of the mean GFP fluorescence intensity (69.27 ± 7.35 and 58.32 ± 6.35 respectively, *P value*: 0.002). But, after 3 weeks, the ovarian sections exhibited highly significant increased fluorescence intensity of GFP-labeled HS-pretreated BM-MSCs (41.05 ± 2.84) more than that of subgroup III-B after 3 weeks (26.37 ± 4.18) with *P value*: 0.001 (Figure 1D, Histogram 1).

Histological Results (Hematoxylin and Eosin-stain)

Group II (Control group): Sections of the ovarian tissue of adult albino rat revealed an outer cortex contained multiple follicles at different stages of maturation and multiple scattered corpora lute. And inner medulla formed of loose vascular connective tissue (Figures 2 A,B). Regular primordial, primary, secondary and graafian follicles were seen (Figures 2 C,D,E,F).

Group III: Experimental group

1-Subgroup III-A (DOX- Treated): showed multiple degenerating cystic follicles with eosinophilic material and many atretic follicles, the medulla contained dilated congested blood vessels (Figures 3 A,B). Degenerated cystic follicles contained eosinophilic fluid and clusters of detached granulosa cells (Figure 3C). Atretic antral ovarian follicles surrounded by congested blood vessels, shrunken oocyte with complete absence of the nucleus and zona pellucida, granulosa cells exhibit pyknotic nuclei and vacuolated cytoplasm (Figure 3D). atretic ovarian follicles with irregular oocyte outline and irregular zona pellucida, the nuclei are irregular eccentric and darkly stained, granulosa cells are disorganized, and some separated from theca cells (Figure 3E). Vacuolated cytoplasm and eccentric or irregular dark nuclei of the oocytes and detached granulosa cells (Figure 3F).

2-Subgroup III-B (DOX+ routine BM-MSCs): The ovarian sections of the rats sacrificed 1week showed multiple degenerating follicles with eosinophilic materials in their cavities, many atretic follicles, but there were multiple corpora lutea, the medulla exhibited dilated congested blood vessels (Figures 4 A,B). Atretic follicle was noticed in the form of irregularity in outline of oocyte accompanied with irregularity of zona pellucida with absent nucleus, granulosa cells are disorganized and widely separated and separated from theca cells (Figure 4C). The rats of the same group sacrificed after 3 weeks showed primordial follicles, primary follicles and multiple corpora lutea but degenerating cystic follicles and atretic follicles, primary follicle revealed widely separated granulosa cells, the medulla still showed dilated congested blood vessels (Figures 4 D,E). Oocyte was regular Regular oocyte with regular zona pellucida (ZP), regular nuclear membrane and prominent nucleolus, some granulosa cells widely separated from each other or separated from the theca cells (Figure 4F).

3-Subgroup III-C (DOX+ HS-pre-treated BM-MSCs): the rats sacrificed 1 week showed the graafian follicle and multiple corpora lutea. Degenerating cystic follicles exhibit eosinophilic material (Figure 5A). Multiple primordial follicles, primary follicle with disorganized follicular cells and atretic follicles with irregular zona pellucida, irregular granulosa cells (Figure 5B). Graafian follicle with regular oocyte, zona pellucida and nucleus and some granulosa cells detached in the antrum or separated from theca cells (Figure 5C). The rats sacrificed after 3 weeks of the same group showed multiple primary follicles, secondary follicles, graafian follicles and corpora lutea (Figure 5D). Regular primordial follicles were noticed with normal oocytes (Figure 5E). Graafian follicles with regular oocyte, zona pellucida and regular granulosa cells with no separation between granulosa and theca cells (Figure 5F).

Counting of the ovarian follicles: Subgroups III-A and III-B showed a significant decrease in the mean number of the primordial follicles. Subgroups III-A exhibited highly

significant decrease in the mean number of preantral and antral follicles and highly significant increase in the mean number of the atretic follicles as compared to control subgroups. Moreover, subgroups III-B showed a significant difference in the mean number of preantral, antral and atretic follicles as compared to control subgroups However, there was no significant difference between subgroup III-C and control subgroups. Subgroup III-C showed significant difference as compared to subgroup III-A and III-B (Histogram 2).

Percentage of the healthy and atretic follicles

After calculating the percentage of healthy and atretic follicles from the equations in all control and experimental subgroups they were summarized in (Table 2, Histogram 3).

Immunohistochemical results (Caspase- 3 stain)

Immunohistochemical examination of the ovarian sections of control subgroups revealed minimal immunoreactivity in the oocytes, granulosa cells and stromal cells. The mean percentage surface area of caspase-3 immunoreactivity was 4.8 ± 2.94 with their immune- score (1) (Figure 6A). Subgroup III-A (DOX-Treated) revealed a highly significant increase mean percentage surface area as it was 91 ± 3.97 as compared with control subgroup with its immune-score was (4) (Figure 6B). Subgroup III-B (DOX+ routine BM-MSCs) sacrificed after 1week the mean percentage surface area was 66.3 ± 11.30 and its immune- score was (3) (Figure 6C). Whereas, in rats sacrificed after 3 weeks of the same subgroup the mean percentage surface area was 30.6 ± 10.21 and its immune- score was (2) (Figure 6D). Both revealed a highly significant increase as compared with control subgroup and a highly significant decrease as compared with subgroup III-A (*P values*: 0.001). In rats of subgroup III-C (DOX+ HS-pre-treated BM-MSCs) sacrificed after 1week, the mean percentage surface area was 62.1 ± 5.09 and its immune- score was (3) there was a highly significant increase as compared with control subgroup and a highly significant decrease comparing with subgroup III-A. But there was no significant difference between it and subgroup III-B for the same period (*P value*: 0.156) (Figure 6E). In rats sacrificed after 3 weeks of the same subgroup, the mean percentage surface area of caspase-3 immunoreactivity was 7.2 ± 2.70 and its immune- score was (1). They revealed a highly significant decrease comparing with subgroups III-A, III-B (after 1& 3 weeks) and III-C (after 1 week) (*P value*: 0.001). But there was no significant difference between it and the control subgroups II (*P values*: 0.563) (Figure 6F, Histogram 4).

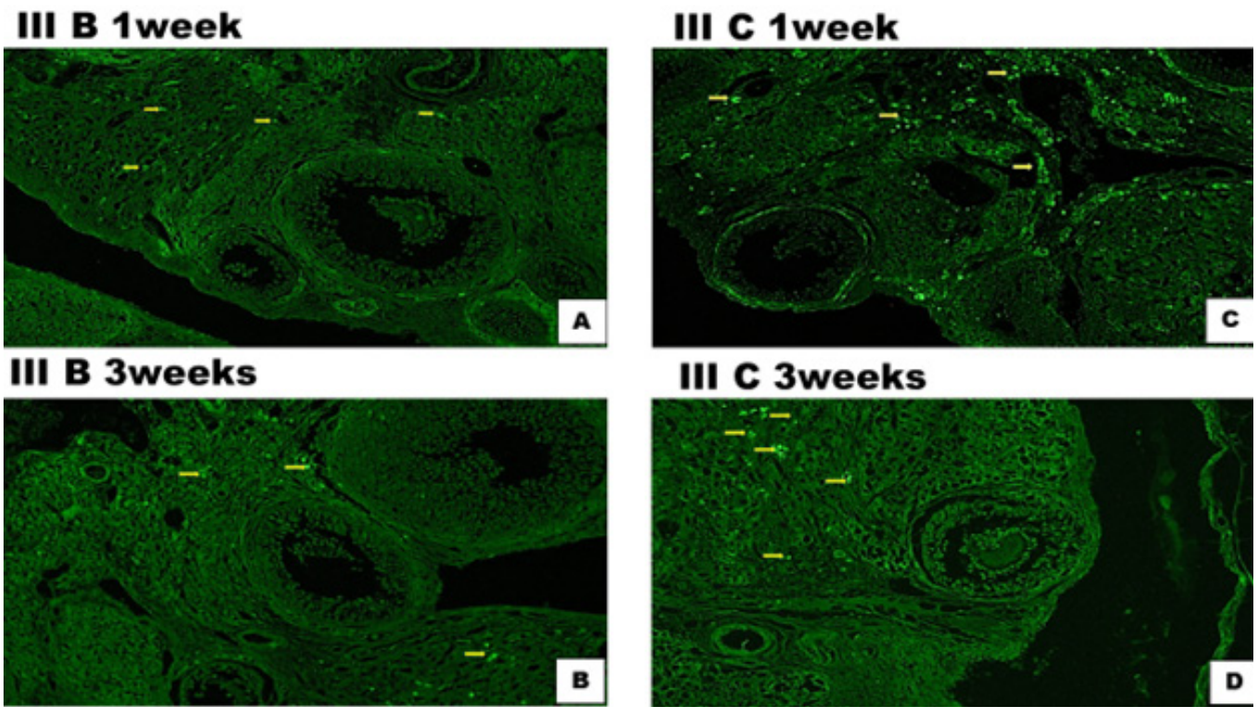


Fig. 1 : GFP by fluorescence microscope X100: Section in the ovary of an adult albino rat of subgroup III-B (DOX+ routine BM-MSCs) sacrificed after 1 week showing multiple GFP-labeled routine BM-MSCs in the ovarian interstitial tissue and surrounding the follicles (arrows) (Fig. 1A). The rats sacrificed after 3 weeks of the same group showing fewer GFP-labeled routine BM-MSCs as compared with those after 1 week scarification in Fig. 1A (Fig. 1B). Subgroup III-C (DOX+ HS-pre-treated BM-MSCs) sacrificed after 1 week showing numerous GFP-labeled HS-pre-treated BM-MSCs (Fig. 1C). The rats sacrificed after 3 week showing multiple GFP-labeled BM-MSCs in the ovarian interstitial tissue and surrounding the follicles (arrows) (Fig. 1D).

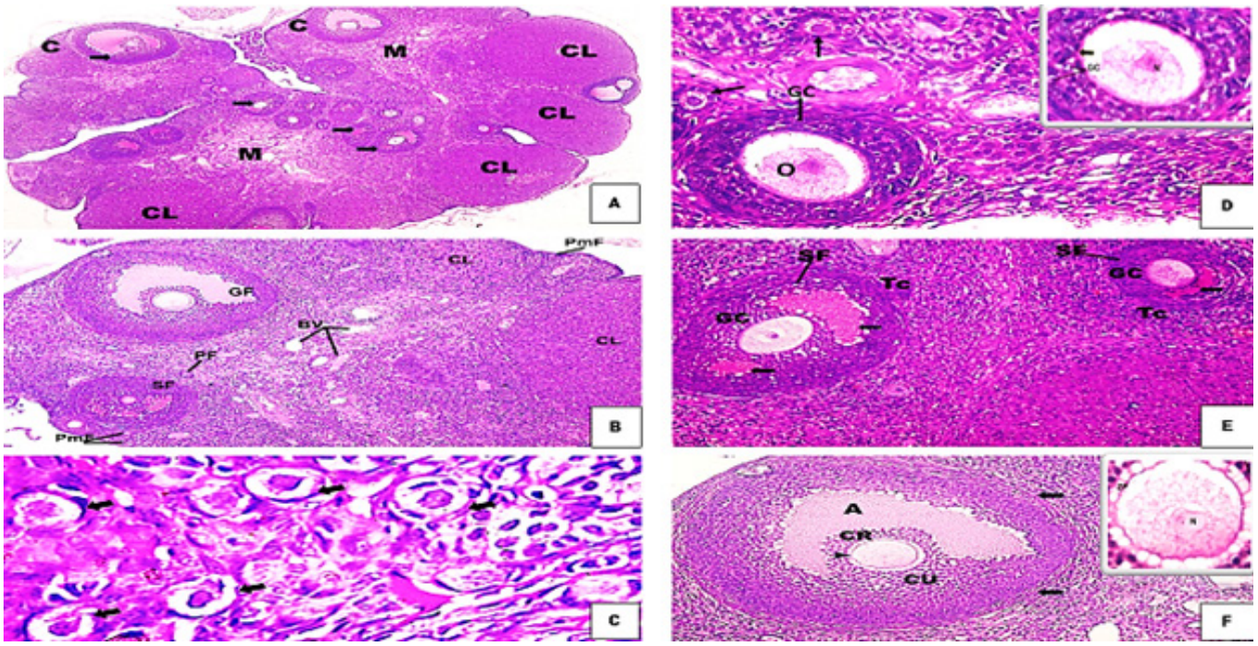


Fig. 2: Group II (control group) (Hx & E.): Section in the ovary of subgroup II-A showing outer cortex (C) and an inner medulla (M). Multiple follicles (arrows) and multiple corpora lutea (CL) (Fig. 2A). Primordial follicles (PmF), primary follicle (PF), secondary follicle (SF), graafian follicle (GF) and corpora lutea (CL). Multiple blood vessels (BV) (Fig. 2B). Multiple primordial follicles each one consists of primary oocytes, surrounded by a single layer of squamous follicular cells (arrows) (Fig. 2C). The primary follicles consist of oocyte (O) and surrounded either by single layer (thin arrows) or stratified follicular granulosa cells (GC) with zona pellucida (thick arrow) (Fig. 2D). Secondary follicles (SF) consist of oocytes surrounded by stratified granulosa cells (GC) and theca cells (Tc), cavities filled with liquor folliculi (arrows) (Fig. 2E). Graafian follicle (GF) is observed with single antrum (A) containing liquor folliculi. Oocyte with central nucleus (N) and zona pellucida (ZP) (arrowhead), corona radiata (CR) and cumulus oophorus (CU) Theca cells (arrows) (Fig. 2F). (A× 40, B, E, F× 200; D x200; C, Inset X1000)

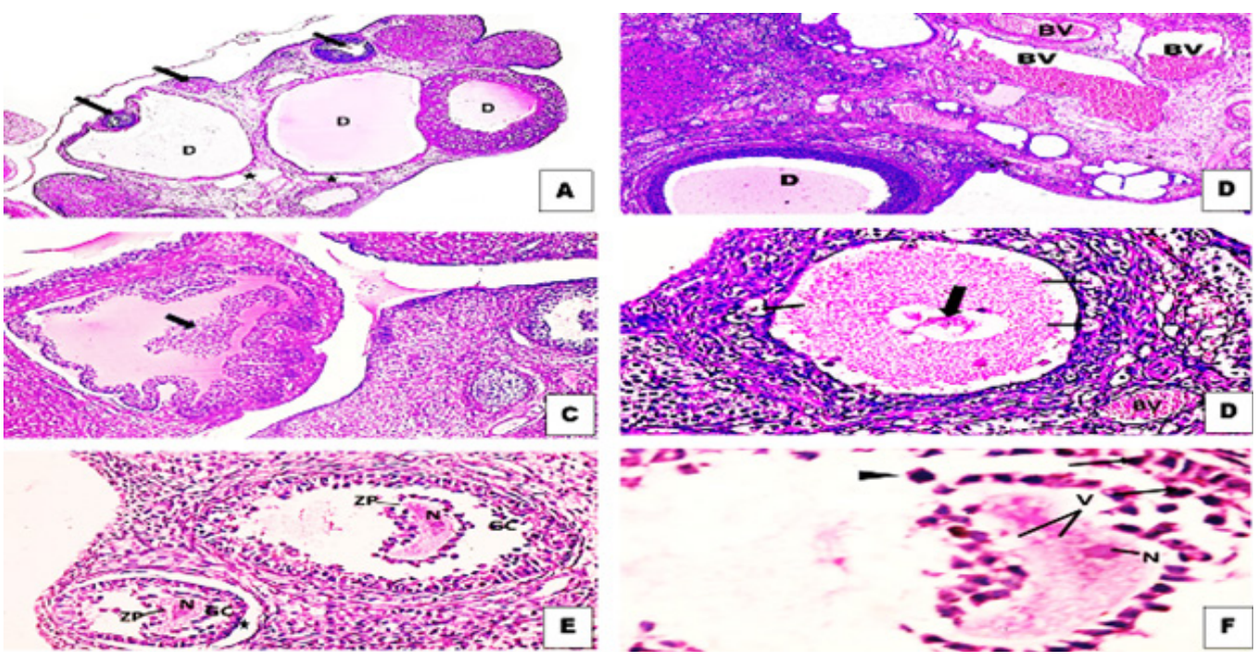


Fig. 3: Subgroup III-A (DOX- treated) (Hx & E.): Section in the ovary of subgroup III-A showing multiple degenerating cystic follicles (D) and many atretic follicles (arrows). (Fig. 3A). Medulla containing dilated congested blood vessels (BV) (Fig. 3B). Degenerated cystic follicle containing eosinophilic fluid and clusters of detached granulosa cells (black arrows) (Fig. 3C). Atretic antral ovarian follicles surrounded by congested blood vessels (BV), shrunken oocyte with complete absence of the nucleus and zona pellucida (thick arrow), granulosa cells exhibit pyknotic nuclei (arrows) and vacuolated cytoplasm (Fig. 3D). atretic ovarian follicles with irregular oocyte outline and irregular zona pellucida (ZP), the nuclei (N) are irregular eccentric and darkly stained, granulosa cells (GC) are disorganized, and some separated from theca cells (Star) (Fig.3E). Vacuolated cytoplasm (V) and eccentric or irregular dark nuclei (N) of the oocytes and detached granulosa cells (arrows) (Fig.3 F). (A× 40, B, C× 100; D, Ex400; Fx1000)

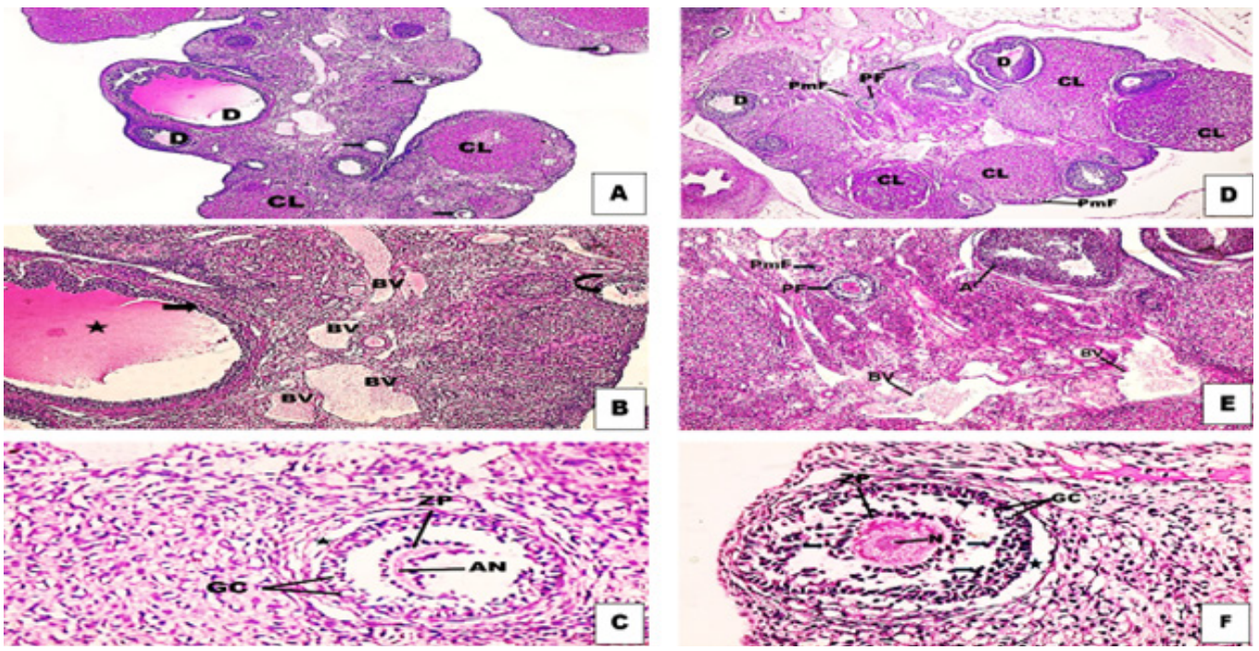


Fig. 4: Subgroup III-B (DOX+ routine BM-MSCs) (Hx & E.): Section in the ovary of subgroup III-B sacrificed after 1 week showing multiple degenerating follicles (D), many atretic follicles (arrows), multiple corpora lutea (CL) (Fig. 4A). Degenerating cystic follicle with disorganized granulosa cells (arrow) and its cavity contains eosinophilic fluid (star), an atretic follicle (curved arrow). The medulla contains dilated congested blood vessels (BV) (Fig. 4B). Atretic follicle reveal irregular oocyte outline accompanied with irregular zona pellucida (ZP), absent oocyte (AN), granulosa cells (GS) are disorganized and widely separated and separated from theca cells (star) (Fig. 4C). The rats sacrificed after 3 weeks showing primordial follicles (PmF), primary follicles (PF) and multiple corpora lutea (CL). Degenerating cystic follicles (D) are seen (Fig. 4D). Primary follicle (PF) with widely separated granulosa cells and The medulla contains dilated congested blood vessels (BV) (Fig. 4E). Regular oocyte with regular zona pellucida (ZP), regular nuclear membrane and prominent nucleolus, some granulosa cells (GC) widely separated from each other (arrows) or separated from the theca cells (star) are still present (Fig. 4F). (A, D× 40, B, E× 100; C, F x400)

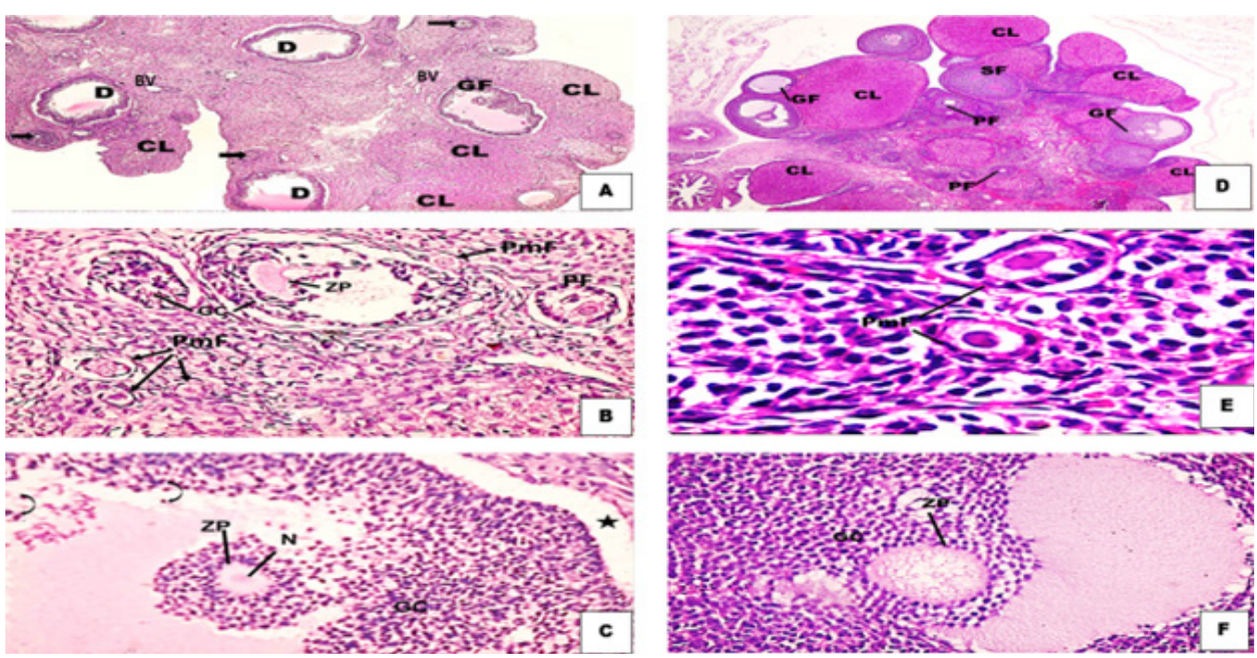


Fig. 5: Subgroup III-C (DOX+ HS-pre-treated BM-MSCs) (Hx & E.): Section in the ovary of subgroup III-C sacrificed after 1 week showing graafian follicle (GF) and multiple corpora lutea (CL). Degenerating cystic follicles (D) exhibit eosinophilic material (Fig. 5A). Multiple primordial follicles PmF(arrows). Primary follicle (PF) with disorganized follicular cells and atretic follicles show irregular zona pellucida (ZP), irregular granulosa cells (GC) (Fig. 5B). Graafian follicle (GF) with regular oocyte, zona pellucida (ZP) and nucleus (N) and some granulosa cells detached in the antrum (curved arrows) or separated from theca cells (star) (Fig. 5C). After 3 weeks multiple primary follicles (PF), secondary follicles (SF), graafian follicles (GF) and corpora lutea (CL) (Fig. 5D). Regular primordial follicles (PmF) (Fig. 5E). Graafian follicles (GF) with regular oocyte, zona pellucida (ZP) and regular granulosa cells (GC) with no separation between GC and TC (Fig. 5F). (A, D× 40, B, E× 100; C, F x400)

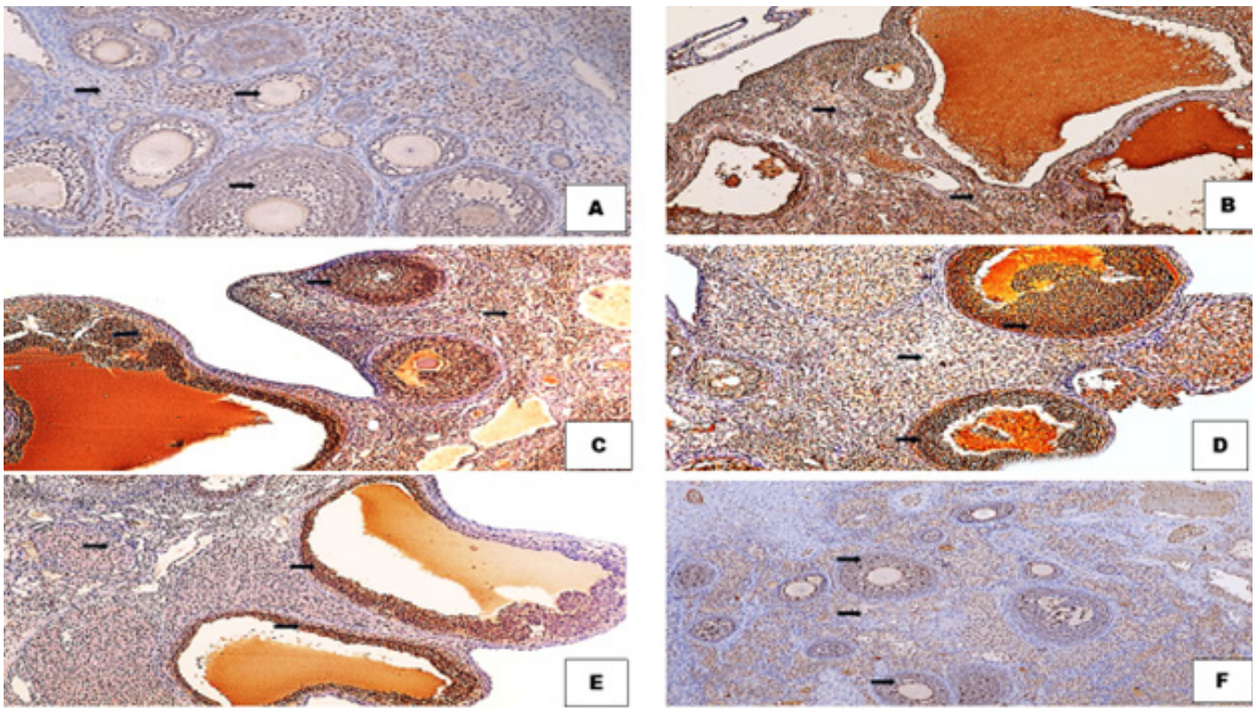


Fig. 6: Caspase-3 immunostaining; X100: Fig. 6: Section in the ovary of subgroup II-A showing minimal expression of caspase-3 immunostaining (Fig. 6A). Subgroup III-A showing obvious positive immunoreaction (Fig. 6B). Subgroup III-B sacrificed after 1week showing strong expression of caspase-3 immunoreactivity (Fig. 6C). After 3weeks showing less obvious immunoreactivity (Fig. 6D). Subgroup III-C sacrificed after 1week showing less obvious expression of caspase-3 immunoreactivity (Fig. 6E). After 3weeks showing minimal immunoreactivity (Fig. 6F).

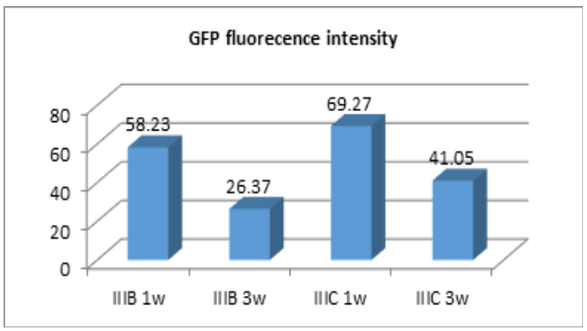
Table 1: The mean serum level of E2 and FSH.

E2 ng/L	II-A	II-B	II-C	III-A	III-B	III-C
Mean ± SD	65.99 ± 2.22	64.72 ± 2.09	63.29 ± 2.91	32.77 ± 1.93	57.22 ± 4.61	61.45 ± 3.21
P1		0.573	0.173	0.001**	0.010*	0.068
P2			0.952	0.001**	0.011*	0.447
P3				0.001**	0.0013**	0.753
P4					0.001*	0.001*
P5						0.045*
FSH IU/L						
Mean ± SD	6.61 ± 0.91	6.75 ± 0.92	7.42 ± 0.70	21.46 ± 1.37	8.99 ± 1.10	7.64 ± 0.85
P1		0.846	0.206	0.001**	0.002*	0.100
P2			0.830	0.001**	0.024*	0.607
P3				0.001**	0.024*	0.992
P4					0.001**	0.001**
P5						0046*

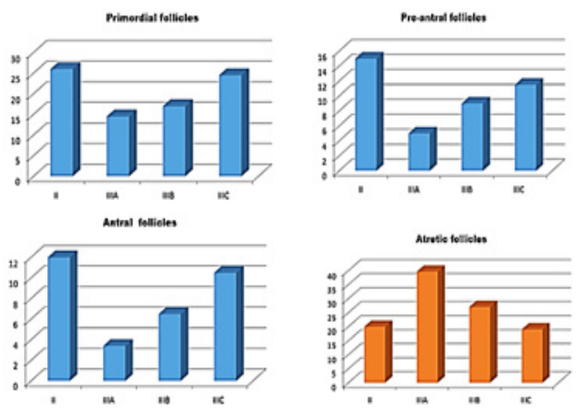
P1 significance with subgroup II-A, P2 with II-B, P3 with II-C, P4 with III-A, P5 with III-B.

Table 2: The percentage of healthy and atretic follicles in all subgroups

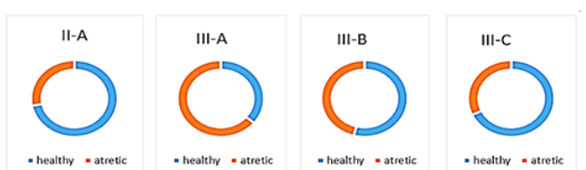
	% of Healthy follicle	% of Atretic follicle
II	71.95	28.05
III-A	36.33	63.67
III-B	53.55	46.45
III-C	68.21	31.79



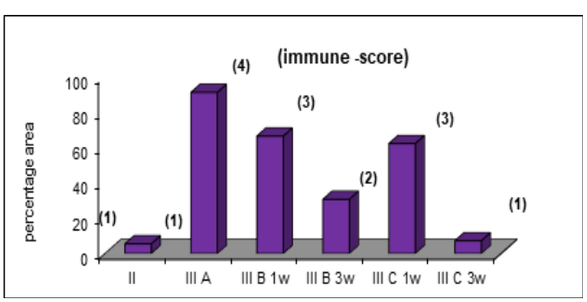
Histogram 1: Green fluorescent protein (GFP) fluorescence intensity of BM- MSCs (routine and heat shock pretreated) in subgroups III-B and III- C,1w= rats sacrificed after 1week ,3w=rats sacrificed after 3weeks.



Histogram 2: The mean number of primordial, pre-antral, antral and atretic follicles.



Histogram 3: The percentage of healthy and atretic follicles in all subgroups



Histogram 4: Caspase-3 percentage surface area and immune- score.

DISCUSSION

Follicle loss and reduced endocrine and reproductive function are features of premature ovarian insufficiency (POI), which affecting women younger than 40[1,2]. Doxorubicin (DOX) is a powerful chemotherapeutic drug that has been used to treat a variety of cancers[37], but it comes with a long list of adverse effects, the most common of which are ovarian dysfunction and POI[17]. In the current study, deaths of rats were noticed only in the experimental group (III) given DOX as the mortality rate was 26.7%. Similarly,[38] found that the group treated with DOX had a significantly higher death rate (42%).

Indicators of ovarian reserve as FSH and E2, was shown to decline with the progression of ovarian insufficiency, as indicated by rising FSH levels and falling E2 levels[3]. The present study found that compared to the control subgroups, rats in subgroup III-A had highly significant decrease in the mean level of E2, and highly significant increase in the mean level of FSH. Also, Xiao *et al*.[39] and Rad *et al*.[40] found that DOX caused significantly disrupted E2 production. These findings coincide with Ben-Aharon *et al*.[41] who reported the incidence of amenorrhea increased dramatically in female cancer patients receiving DOX, suggesting depletion of growing follicles and decreased E2 level. This could be explained by the interactive the hypothalamic-pituitary-ovarian (HPO)axis feedback. In POI there is inadequate secretion of E2 as a result of decreased quality or quantity of follicles which results in a preferential rise in FSH[42]. Moreover, said *et al*.[34] suggested that granulosa cells apoptosis induced by doxorubicin results in a decrease in E2 levels, interrupting the negative feedback mechanism and causing an uncontrolled increase in FSH levels.

In both subgroups III-B and III-C there was a highly significant increase in the mean serum level of E2 and a highly significant decrease in the mean serum level of FSH as compared to the subgroup III-A. Moreover, the serum E2 level was significantly higher in subgroup III-C than that of subgroup III-B, while the FSH concentration was significantly lower in subgroup III-C than that of subgroup III-B indicating the beneficial effect of heat shock pretreated BM- MSCs on the ovarian function after DOX. This observation agreed with Chen *et al*.[22], who observed that in the heat shock pre-treated group the serum E2 level was higher and FSH level was lower than the MSC group. They revealed that heat shock pre-treatment of MSCs was more effective in improving the damage of the ovarian structure and endocrine function after chemotherapy and attributed this to the restoration of the estrous cycle in some rats following transplantation of heat shock-pretreated MSCs into chemotherapy-damaged rat ovaries

In the present work routine or HS-pretreated BM-MSCs were traced after injection by green fluorescent protein (GFP). It is reported to become the marker of choice for tracing of transplanted MSCs as it is safe, nontoxic and has high sensitivity and specificity[27]. The rats sacrificed

after 1 week in subgroup III-B (DOX+ routine BM-MSCs) revealed multiple GFP-labeled BM-MSCs in the ovarian interstitium with their mean intensity was 58.32 ± 6.35. Whereas in the rats sacrificed after 3 weeks of the same subgroup there was a highly significant decrease of the mean GFP fluorescence intensity as it was 26.37 ± 4.18. These findings agree with Liu *et al*.[20] who traced GFP-labeled BM-MSCs in the ovarian tissue up to 30 days after injection. In contrast Wang *et al*.[23] found no ovarian fluorescence signals at the day 21 following injection. Moeinabadi-Bidgoli *et al*.[12] acidosis, hypoxia, oxidative stress, inflammation, and nutritional deficiency are some of the microenvironmental alterations that might impact the survival of stem cells. Li *et al*.[8] discovered that MSCs' ability to repair damaged ovaries is made possible by a multi-step process including migration and homing, which is explained by the unique traits of various MSC types and various methods of labeling them. Human umbilical cord stem cells Song *et al*.[43] and menstrual blood stem cells Noory *et al*.[44] may continue to develop into granulosa cells after surviving in the ovarian stroma for two months following transplantation. Despite this, some research has shown that MSCs can enhance ovarian function by localizing to the ovarian matrix, secreting a variety of cytokines, and not differentiating directly into oocytes[29,45].

In the present study heat shock preconditioning increased the traced GFP-labeled BM-MSCs. Subgroup III-C (DOX+ HS-pre-treated BM-MSCs) after 1 week showed a significant increase of the mean GFP fluorescence intensity of BM- MSCs (69.27 ± 7.35) as compared with subgroup III-B after the same period. On the other hand, subgroup III-C after 3 weeks revealed a highly significant increase of the mean GFP fluorescence intensity of BM-MSCs (41.05 ± 2.84) as compared with subgroup III-B after the same period (26.37 ± 4.18). These results could be explained by Qiao *et al*.[46] and Kim *et al*.[47] who observed that the increased migration of transplanted cells to the injury site and the increased expression of GFP in heat shock pre-treated MSCs are the result of the elevated expression of homing-related genes (CCL7, CXCR-4). While BM-MSCs tagged with GFP were not found within the follicles themselves, they were found in the interstitium and around the ovary in this study. Based on these findings, it seems that BM-MSCs could engraft in the interstitium instead of immediately differentiating into oocytes and granulosa cells, which would have restorative effects on follicle growth[18,20,23].

In the present study, H& E-stained rat ovarian sections of subgroup III-A (DOX-treated) showed multiple degenerating cystic follicles and many atretic follicles. These findings were explained by Roti Roti *et al*.[48] who described how doxorubicin induces DNA double-strand breaks by intercalating into the minor groove between guanine-cytosine base pairs, preventing transcription. Degenerated cystic follicles contained eosinophilic fluid and clusters of detached granulosa cells, they were surrounded by congested blood vessels a Similar finding were noticed in the studies of Xiao *et al*.[39] and Wang *et al*.[49] as they reported distorted, shrunken, or extruded oocyte and degenerated granulosa cell layers in antral stages of ovaries in DOX-treated mice. Atretic ovarian follicles was observed, they exhibited irregularity in outline of oocytes and zona pellucida with eccentric irregular darkly stained nuclei. This observation coincides with Chen *et al*.[50], who reported that the oocyte from the ovaries cultured in a medium supplemented with DOX associated with loss of oocyte shape and zona pellucida with structural alterations.

Atretic antral ovarian follicles with shrunken oocyte with complete absence of the nucleus and zona pellucida, granulosa cells with pyknotic nuclei vacuolated cytoplasm, detatched and separated from theca cells. Andersen *et al*.[51] revealed increased granulosa cell death in the growing follicles and proved reduction of endothelial cell proliferation and disruption of surrounding capillaries. Moreover, de Assis *et al*.[52] reported DOX accumulated the ovarian follicles resulting in disorganized granulosa cells, detached from the basement membrane and retracted oocyte, pyknotic nuclei. Many congested blood vessels observed around some degenerated follicles could be explained by neovascularization induced by DOX in the ovarian cortex[17,41]. Moreover, it could be due to damage of the ovarian microvasculature and increased production of reactive oxygen spices by DOX resulting in death and loss of stromal cells[52,41].

Examination of H& E-stained ovarian sections of subgroup III-B (DOX+ routine BM-MSCs) sacrificed after 1week, the cortex contained multiple degenerating cystic follicles with eosinophilic materials in their cavities. Many atretic follicles with irregular oocytes outline were observed, but there were multiple corpora lutea. Medulla exhibited dilated congested blood vessels. Moreover, rats sacrificed after 3 weeks of same subgroup the cortex contained primordial, primary follicles and multiple corpora lutea but degenerating cystic follicles and atretic follicles were still present. These findings coincide with Song *et al*.[43] who reported that the key for MSCs efficacy is their homing in the target organ for a long time. Li *et al*.[53] demonstrated that some of mesenchymal stem cells have a prolonged half-life after homing to the target organ, leading to a therapeutic impact that lasts for long time. Little improvement in subgroup III-B in this work might be due to MSCs loss after transplantation. But, they could repair ovarian damage to some extent after 3 weeks by inhibiting granulosa cells apoptosis with partial recovery of ovarian damage. Therefore, improving the viability of BM-MSCs in ovarian tissue is essential for enhancing their long-term efficacy[26,50].

In this research, subgroup III-C (DOX+ HS-pre-treated BM-MSCs) sacrificed 1 week after injection of heat shock pre-treatment BM-MSCs the cortex contained many primordial follicles compared to subgroup III-B. Also, primary follicle, graafian follicles and corpora lutea were observed. The graafian follicles exhibited regular oocytes, zona pellucida and nuclei. Granulosa cells were regularly

arranged around the oocyte, but others were still detached in the antrum or separated from theca cells, Moreover, rats sacrificed after 3 weeks revealed multiple primordial, primary, secondary, graafian follicles and corpora lutea with few atretic follicles. Multiple graafian follicles were observed in the cortex exhibiting regular oocyte, zona pellucida and granulosa cells around follicular antrum that contained liquor folliculi and surrounded by theca cells with no separation between them. These findings coincide with those of Chen *et al*.[22] and Choudhery *et al*.[54] He elaborated on the idea that pre-treatment with heat shock might enhance the transplanted BM-MSCs' vitality, proliferative capacity, anti-apoptotic ability, and tissue repair potential. Wang *et al*.[26] shown that HS-pretreated BM-MSCs work by increasing expression of heat shock proteins 70 and 90 and decreasing autophagy, which in turn prevents and treats cisplatin-induced POI. Whereas, Lv *et al*.[13] found that HS pretreatment improved MSCs' immunoregulatory function, which in turn improved their ability to repair acute lung damage, significantly increasing their therapeutic potential. Optimizing the treatment result for premature ovarian failure requires enhancing the homing efficiency of MSCs[10,45]. Thus, the current work suggests that pre-treating BM-MSCs with heat shock could enhance their therapeutic potential by increasing their homing and survival rates, as well as their migratory capacity to the injury site[46].

The current study found that subgroup III-A, had a significantly lower mean number of primordial follicles compared to control subgroup, this finding agrees with Ben-Aharon *et al*.[41] and Samare-Najaf *et al*.[55], He explained loss of primordial follicles during chemotherapy as a result of neovascularization and damaged cortical blood vessels. They hypothesized that local ischemia caused by blood vessel congestion, wall thickening, and lumina obliteration destroyed the area of normal ovarian cortex and caused the loss of primordial follicles. Moreover, Morgan *et al*.[56] and Spears *et al*.[57] found that oocytes may have an accelerated natural aging process as a result of direct or indirect cytotoxic effects, which may hinder folliculogenesis in some primordial follicles, leading to primordial follicle loss in response to DOX or cisplatin.

On the other hand, in the current study, morphometric counting of other ovarian follicles in subgroup III-A revealed highly significant decrease in the mean number of the pre-antral and antral follicles and highly significant increase in the mean number of the atretic follicles as compared to the control subgroups. This finding was explained by Samare-Najaf *et al*.[17], Rad *et al*.[40] and Nishi *et al*[58], who reported that ovarian damage, follicular cells apoptosis and cell proliferation is inhibited by the alkylating toxic actions of doxorubicin. However, Wang *et al*.[49] found no statistically significant decrease in DOX-treated primary follicles, which may indicate that some primordial follicles were activated and recruited to become primary follicles.

Subgroup III-B, which received routine BM-MSC, continued to show significantly lower mean number of primordial follicles compared to the control subgroups. there was no significant difference between the control groups and the subgroups who received injections of HS pretreated BM-MSCs (subgroup III-C). Similar findings were observed in the works of Chen *et al*.[22] and Ebrahim *et al*.[59] They showed that MSCs treatment did not entirely prevent quiescent follicles from the chemotherapy-induced ovarian damage, but the HS pre-treated group had more quiescent primordial follicles than the non-treated MSCs group, MSCs treatment did not entirely prevent quiescent follicles from the chemotherapy-induced ovarian damage.

On the other hand, as compared to the control subgroups, subgroup III-B exhibited significant decline in the average number of pre-antral and antral follicles and increase in the average number of atretic follicles. When comparing subgroup III-C to the control subgroups, the mean number of pre-antral, antral, and atretic follicles was not significantly different. These results coincide with those of Ebrahim *et al*.[59] and Wei *et al*.[60] who proposed that MSCs have a restricted function for rescuing follicles suffering early atresia with partially restoring ovarian function and partially counteracting chemotherapy's effects.

In this work, apoptosis was assessed using an immunohistochemical stain of caspase-3 positivity. The mean % surface area of caspase-3 immunoreactivity in subgroup III-A (DOX-treated) was significantly higher than the control subgroups, with an immune-score of[4]. This finding is similar to that reported by Wang *et al*.[49]and Morgan *et al*.[56] they found after DOX treatment, caspase-3 signals had significantly risen which indicated apoptosis in granulosa cells and oocytes. Doxorubicin, according to their theory, hinders DNA replication and cell division by blocking topoisomerase enzymes. Moreover, Mousa *et al*.[61] the DOX group may have experienced heightened oxidative stress due to an increase in free oxygen radical generation, which may have contributed to their higher rate of apoptosis.

Following the injection of routine BM-MSCs (subgroup III-B) in this study, rats euthanized one week later showed strong caspase-3 immunoreactivity; the immunological score for these sections was[3]. Whereas, in rats of the same subgroup sacrificed after 3 weeks revealed a highly significant increase as compared with control subgroups and a highly significant decrease as compared with subgroup III-A with its immune- score was[2]. This current result is consistent with Fu *et al*.[62] who found that MSCs can upregulate Bcl-2 protein and other cytokines, which inhibits apoptosis caused by chemotherapy of granulosa cells., Other authors however, have shown that transplanted MSCs only partially repair chemotherapy-induced POI because to the death of the transplanted cells[22,26]. Elfayomy *et al*.[30]. found that mesenchymal stem cells could rescue preexisting oocytes by reestablishing the normal epithelial arrangement and improving the ovarian niche by regulating the expression of cytokeratin 8/18,

transforming growth factor β (TGF-β), and proliferating cell nuclear antigen (PCNA) in the ovary. Bao *et al*.[63] found that transplantation of BM-MSCs resulted in reduced apoptosis of ovarian cells, as indicated by decreased levels of Bax, p53, and p21.

On the other hand, subgroup III-C (DOX+ HS-pre-treated BM-MSCs) sacrificed after 1 week its immune-score was[3]. In rats sacrificed after 3 weeks of the same subgroup revealed a highly significant decrease comparing with subgroups III-A, III-B. But there was no significant difference between it and the control subgroups its immune- score was[1]. The specific mechanisms by which HS pre-treatment inhibits the apoptosis of BM-MSCs are still unknown. McGinley *et al*.[64] suggested that heat shock proteins (HSPs) play a role in inhibiting apoptosis through heat shock pre-treatment. Choudhery *et al*.[54] observed that elevated levels of HSP27, 70, and 90 reduced apoptotic cells and promoted the proliferation of MSCs. Furthermore, HSPs are involved in blocking various apoptotic pathways. Apaf-1 may be bind to HSP27 and HSP90, which inhibits apoptosome formation and mitochondrial apoptotic pathway activation. On the other hand, caspase-mediated apoptosis is inhibited by HSP70 through its interaction with apoptosis-inducing factor[46].

CONCLUSION

From the present study, it could be concluded that heat shock pre-treated BM-MSCs had a great effect in repairing the degenerative changes associated with DOX induced premature ovarian insufficiency more than routine BM-MSCs. Also, HS pre-treatment effectively improved the proliferative capacity, homing and anti-apoptotic ability of BM-MSCs.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دور الخلايا الجذعية الوسيطة للنخاع العظمى المعالجة مسبقاً بالصدمة الحرارية مقابل الغير معالجة في إصلاح قصور المبيض المبكر المستحث تجريبياً بواسطة الدوكسوروبيسين

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الخلفية: قصور المبيض المبكر هو واحد من أخطر المشاكل التي تهدد الصحة الإنجابية للنساء في سن الإنجاب الطبيعي.
الهدف من العمل: : كان الهدف من هذه الدراسة هو إلقاء الضوء على تأثير الخلايا الجذعية الوسيطه للنخاع العظمى المعالجة مسبقًا بالصدمة الحرارية مقابل غير المعالجة لإصلاح قصور المبايض المبكر المستحث تجريبيا بواسطة الدوكسوروبيسين .
طرق ومواد البحث: تم استخدام ستة وخمسين أنثى بالغة من جرذان البيضاء وتم تقسيمها إلى ثلاث مجموعات المجموعة المانحة للخلايا الجذعية تضمنت ١٠ جرذان, المجموعة الضابطة تضمنت ١٦ جرذا المجموعة التجريبية (٣٠ جرذا) تلقى كل جرذ (١ مل) ٢,١ ملغم / كغم من وزن الجسم دوكسوروبيسين جرعه كل ثالث يوم بواسطة سبع حقن داخل الصفاق ، تم تقسيم هذه المجموعة إلى ثلاث مجموعات فرعية (كل منها ١٠ جرذان) المجموعة الفرعية III-A(الدوكوروبيسين),المجموعة الفرعية III-B(الدوكوروبيسين+ الخلايا الجذعية الوسيطة للنخاع العظمى العاديه) والمجموعة الفرعية III-C (الدوكوروبيسين+ الخلايا الجذعية الوسيطة للنخاع العظمى المُعالجة مسبقًا بالصدمة الحرارية) وتم استخدام البروتين الفلوري الأخضر لتمييز وتتبع الخلايا الجذعية الوسيطة ، وتم قياس مستويات E٢ و FSH ، تم التضحية بالجرذان وتجهيز عينات من المبيض لإجراء الدراسات النسيجية والهستوكيميائية المناعية والقياسية.
النتائج: كشفت هذه الدراسة أن وظيفة المبيض في الجرذان التي تلقت الخلايا الجذعية المعالجة مسبقًا بالصدمة الحرارية عن زيادة ذو دلالة احصائية في مستوى هرمون E٢ وانخفاض ذو دلالة احصائية في مستوى FSH أكثر من الجرذان التي تلقت الخلايا الجذعية العادية. وقد ظهر أن المعالجة المسبقة بالصدمة الحرارية تزيد من توجيه الخلايا الجذعية الوسيطة للنخاع العظمى والذي تم إثباته من خلال الزيادة الملحوظه للخلايا الجذعية المميزه بالبروتين الفلوري الأخضر في المجموعة الفرعية III-C مقارنة بالمجموعة الفرعية III-B وكان هناك تحسن نسيجي ملحوظ في مقاطع المبيض وانخفاض ذو دلالة احصائية في موت الخلايا المبرمج فى الجرذان التي تلقت الخلايا الجذعية المعالجة مسبقًا بالصدمة الحرارية أكثر من الجرذان التي تلقت الخلايا الجذعية العادية والذى يتضح من النشاط المناعى لCaspase-٣ .
الاستنتاج: كان للخلايا الجذعية الوسيطه للنخاع العظمى المعالجة مسبقًا بالصدمة الحرارية تأثير كبير في تقليل التغيرات التنكسية المرتبطة بقصور المبايض المبكر المستحث تجريبيا بواسطة الدوكسوروبيسين أكثر من الخلايا الجذعيه العاديه.