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# Comparative effect between Mesenchymal Stem Cells (MSCs) and Clomiphine citrate on induced premature ovarian failure (POF) in mice

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**Abstract**
The present study was conducted to do a comparative between MSCs and Clomiphene citrate on induced POF in mice in College of Veterinary Medicine/ University of Baghdad from September 2016 to September 2017. 4-vinylcyclohexene diepoxide (80 mg/kg) B.W was used to induce POF. Forty females mice were separated into four groups, the first group (T1) was control, second group (T2) was induced and handled with phosphate buffer saline, third group (T3) was induced and handled with MSCS (25x $10^6$cell / kg) B.W, fourth group (T4) was induced and treated with Clomiphine citrate (0.1 mg/kg) B.W. The results of T3 showed regulation in hormones with normalize the glutathione peroxides, superoxide dismutase levels, fertility index% with a significant increase in serum vascular endothelium growth factor as compared with T2 and T4. Histopathological result of T3 showed regular outline of growing ovarian follicles as compared with T1, T2 and T4. This study concluded that the MSCs had inhibitory effect to POF without side effect whereas Clomiphene citrate was attributed in healing of POF with many side effects.

**Keywords:** Mesenchymal Stem Cells, Clomiphene citrate, Premature Ovarian Failure.

## 1. Introduction
Stem cells had a remarkable potential that promised for supplying cells to treat debilitating of several cell kinds in the body, Stem cells (SC) also served as a sort of repairing diseases without any side effect. Stem cells are not specialized cells in the body that had the capacity to differentiate into different kinds of body tissues with particular functions, in addition to Mesenchymal stem cells had the capacity to distinguish into multiple of tissues such as hepatocytes, osteoblasts and ovarian tissue. Stem cell very promised for providing cells to treat incapacitating diseases resembling type-1 diabetes, Parkinson's disease, end stage liver diseases, spinal cord damage, Alzheimer's disease, cardiac diseases, burns, rheumatoid arthritis, stroke, osteoarthritis and cancer [1]. Stem cell transplantation could be helped in the recovering of ovarian dysfunction in animals suffered from premature ovarian failure, by improving ovarian function and inhibiting stromal cell apoptosis during the excretion of some paracrine factors and stannio-calcin -1, in addition, to reinstate ovarian folliculogenesis with steroid hormones creation [2]. Premature ovarian failure can be defined as a major ovarian defect described by absent menarche or as the cessation of ovarian role early which is characterized by the presence of oligomenorrhea or amenorrhea, absent estrus cycle, unregulated of gonadotropins, increase free radicals, sexual dysfunction and hypothyrodism. The etiologies of POF occurred from genetic defects; iatrogenic damages, metabolic factors, autoimmune ovarian damage, radiotherapy and enviro-nmental toxins such as 4-vinylcyclohexene diepoxide (VCD) [3]. Several drugs used to treat premature ovarian failure such as GnRH antagonists, Tamoxifen, letrozole, Gonadotrophin, progestin and Clomiphene citrate, but these drugs have several side effect [4]. Clomiphene citrate is a non-steroidal agent and usually is considered as the first choice fertility drug for inducing ovulation in animals [5]. The side effect of Clomiphene citrate lead to invasive ovarian tumor, multiple pregnancy, increase free radicals with decrease oocyte quality, weight gain, swelling of the legs or hands, shortness of breath and abdominal pain [6]. So the project of this study was founded to treat the premature ovarian failure without any side effect.

## 2. Materials and Methods

### 2.1. Isolation and culturing MSCs

The procedure technique was done according to Rochefort *et al*., [7]. Mesenchymal stem cells carefully were collected from the bone marrow of femurs and tibiae of adult male mice by reddening the femurs and tibiae with 10 ml Ross Well Park Memorial Institute medium (RPMI, GIBCO/ Italy), after that the perched cells were put in centrifuge at 200g for 5 minutes, to obtain cell pellet and remove the supernatant (fat with serum layers). The cell pellet re suspended in 10 ml RPMI1640 media and put in 50 $cm^2$ cultures flasks (falcons) and then added 1ml of fetal calf serum (10%) (GIBCO/ USA) with penicillin with streptomycin (1%). Cells were kept on 37 ˚C in 5% moistened CO2 for 72 hrs. The large colonies of adherent cells were developed (80% - 90% confluence) which examined under inverted microscope, while the non-adherent hematopoietic cells were removed, the consequential cultures were pointed as first-passage of culture. To re sub culture the adherent cells were de attached by washing the cultures two times by phosphate buffer saline and 2 ml 0.25% trypsin was added to cells with 1 mm Ethylene Diamine Tetra Acetate about five minutes at 37 ˚C. Subsequent the cells were dissociated to single cells suspension, 10 ml of culture media with 1 ml of 10% fetal calf serum was added to culture flask (Falcon) of cells suspension and incubated at 37 ˚C for another 72hr. The resultant cultures were pointed to as second-passage culture. The MSCs were cultured to the third passage. MSCs in culture were inspected daily to observed the growth of cells and the media was changed every three day, in addition the daily photographs was taken by using inverted microscope at magnification 40X, the pictures of MSCS growth in third passage characterized by their adhesiveness and fusiform with a spindle-like shape. The Anti-CD44 antibody (kit No: ab157107 and ab64261) was used to detect MSCs according to [8].

### 2.2. Determination of cell count and viability

Mesenchymal stem cells (MSCs) detached from the falcon surface with trypsin-versene and counted by double Neubauer ruling counting chamber (slid chamber). Trypan blue stain was intended via dissolving 0.1gm of trypan blue dye powder in 100 ml of Phosphate Buffered Saline (PBS), this stain was used for determination and counting of the viable cells. Amount of 0.5 ml of cell suspensions was added to 0.5ml of the trypan blue stain in sterile test tube and incubated at 37 $^0$C for thirty minutes. 0.02ml of the mixture was placed in counting hemacytometer chamber and then but beneath light microscope at magnification powers (40X). By using the following equation counting cells concentration (cell/ml) = total cells count x dilution factor x $10^4$/ number of squares (five squares). MSCs viability was measured according to equation viability of cell= number of non-stained cells / total number of cells x100, the dead cells were stained by the dye whereas the living were not stained by the dye. When quantity of stained cells more than 30% the samples were get rid and re subculturing of cells were made by growth media [9].

### 2.3. Induction of Premature ovarian failure in mice

4-vinylcyclohexene diepoxide was used to persuade Premature ovarian failure, thirty five female animals were given I/P daily dose of 4-vinylcyclohexene diepoxide (80mg/kg) B.W. which dissolved with sesame oil, the Premature ovarian failure was developed after 15 days from the first injection [10]. After the end period of induction five female mice were taken randomly and scarified to study the histopathological changes of ovaries and measurement the hormones (FSH, LH, estrogen and progesterone) to ensure the induction of Premature ovarian failure was induced.

### 2.4. Experimental design

Forty female mice were separated into four identical groups, each group containing 10 female mice and the periods of treatment was 30 days as follows: The animals of the first group (T1) were not induced POF and left without any treatment (negative control group). The animals of the second group (T2) were POF induced and treated with phosphate buffer saline by using stomach tube given orally daily (positive control group). The animals of the fourth group (T3) were POF induced and treated with Mesenchymal stem cells at dose 25x $10^6$cell /kg. B.W given I/P once time weekly. The animals of the third group (T4) were POF induced and treated by daily dose (0.1mg/kg) B.W of Clomiphine citrate given orally by stomach tube.

### 2.5. Ratio of ovary weight measurement

The ratio of ovaries organs was calculated according to equation reported by [11] as following: Ratio of ovaries = weight of ovaries/ body weight x100.

### 2.6. Determination of estrogen, progesterone, LH, FSH, antimull-erian hormone and Serum vascular endothelial growth factor levels

The quantitative analysis of circulating serum levels of progesterone, estrogen, LH, FSH, anti mullerian hormone and vascular endothelium growth factor were done by employing an enzymes associated with immunosorbent assay (ELISA Kits). The analyses were done in Clinical Laboratory of Wahj DNA Laboratory/Baghdad - Al-karada.

### 2.7. Determination of Glutathione peroxidase and Superoxide dismu-tase in serum.

The serum levels of superoxide dismutase and Glutathione peroxidase were performed according to the details given in Biodiagnostic kits instruction, SOD was measured spectrophotometrically at 560 nm with referencing to the technique of Nishikimi *et al*, [12], the technique debended on the capacity of SOD to restrain the phenazine- methosulphate arbitrated to the reduction in dye of nitroblue tetraazolium, while the GPX was resoluted spectrophotometrically according to the methods of Paglia and Valentine, [13], the evaluation of GPX depended on the oxidation of NADPH and GSH by utilizing Glutathione R eductase and standardizing the reduction in absorbance at 340 nm.

### 2.8. Determination of fertility index and number of embryos

After end the experiment, five animals from each group were taken and the estrous phase was detected by vaginal smear, then each two female put with one male for mating and the vaginal smears was taken to insure from the presence of sperms, after one week from mating the female rats were sacrificed to determine the number of embryos. (Fertility index: number of pregnant/ total number of mated female rats x 100). This parameter was measured according to [14].

### 2.9. Histopathological method

After the end of treatment, mice were anesthetized via diethyl ether**,** mice were sacrificed and samples of ovaries have been excised and also clean off from the involved connective tissue and fat, then preserved in 10% formalin for fixation,

processed regularly in histokinette, cutoff at 5mm density via microtome and stained with Haematoxylin with eosin and then tested underneath light microscope [8].

### 2.10. Detection of MSCs by Immunohisto-chemical staining for the CD44

After paraffin slides were prepared as mentioned previously the following methods were used according to [15]. The slides were deparaffinized, rehydrated, immersed in xylene (3 minutes and twice times) and xylene with 100% ethanol (3 minutes).The slides were re hydrated in a declining ethanol series and mingled with distilled water (100%, 100%, 95%, 75% and 50%) after this step. The slides were rinsed in cold tap water and put in 100% retrieval buffet citrate in ph 6.0, then the slides were put in the water bath about 5 minute at 95c°. Slides were desiccated at room temperature and a drop of hydrogen peroxide was put in each slide for 30 minute to block the endogenous activity of peroxidase, then all slides were washed with (PBS). Drop of Protein blocker was put in each slide for 30 minute. After that the drop of primary antibodies was added in each slides and left over night. In the second day, the slides were washed with phosphate buffer, the secondary antibody (biotinylated goat antirabbit) was added on each slides for 30 minute and the slides were rinsed three time with PBS. The drop of strepavidin peroxidase was added in each slide for 30 minute, then the slides also rinsed three time with PBS. The situates of antibody binding were visualized following added one drop of DAB stain in each slide. At the end, the slides were stained with hematoxyline stain for 10 minute, washed and dried at room temperature and put under microscope to read.

### 2.11. Statistical analysis

The statistical analysis system- one way was employed to results of all parameters of study. Least statistical variation LSD test at ($P<0.05$) was applied to statistical compared between means in this study [16].

## 3. Results

### 3.1. Isolation and separation of BM-MSCs

The results of culturing in first day showed most of BMSCs were floated as thin arrows in culture medium under inverted microscopy as in (Fig. 1-A), while in second day Mesenchymal Stem Cell were observed adhering to the culture flask thinly and this cells revealed a spindle-like shape (Fig. 1B), whereas in third day the cells displayed a large colonies and began to proliferate in culture media, in addition to a spindle-like shape, under inverted microscope (Fig. 1-C). Whenever the time of cells growth was increased the colonies of cells gradually extended in their size with each adjacent ones unified with each other as in six day of culturing (Fig. 1-D). The cells nearly ceased in their proliferation, adhesiveness and fusiform with a spindle-like shape at nine day (Fig. 1-E). The results of immunophenotypic analysis MSCS at third passage showed that the BM- MSCS adherent cells were positive reaction for CD44 marker and strongly marked with deep brown color DAB blemish, the cells were observed under inverted microscope (X100). (Fig. 1-F).

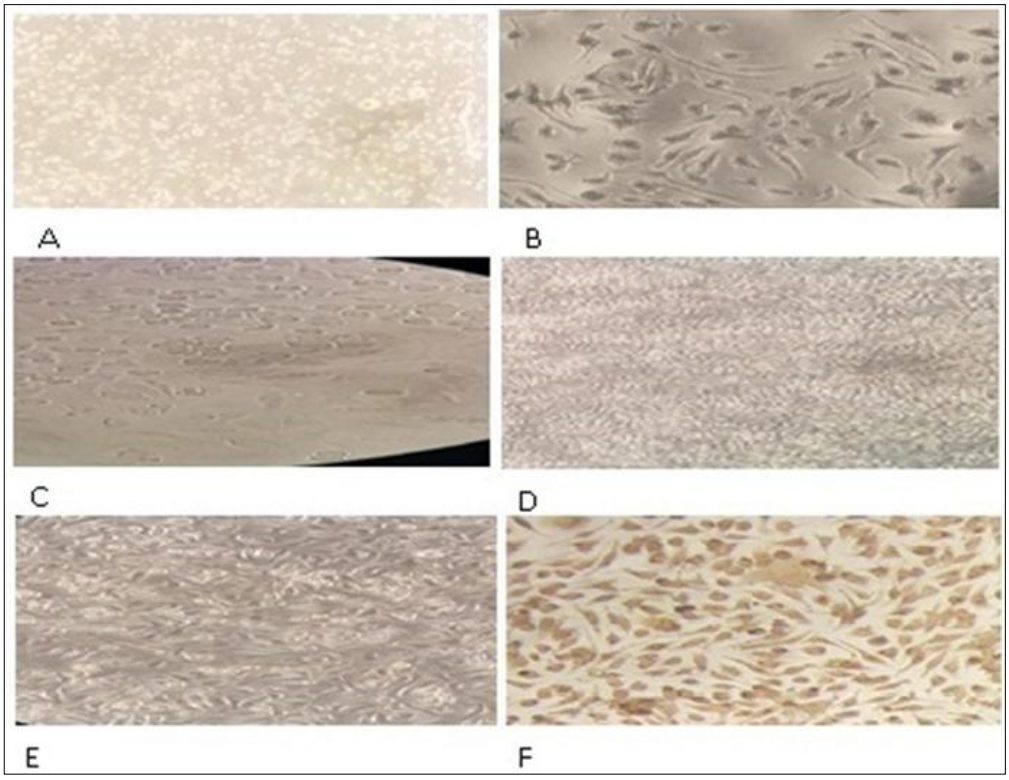


**Fig 1:** The morphology of BM-MSCs at first day of culturing appeared as floating in culture flask (A), at second day the MSCs are revealed a spindle-like shape (B), at third day the cells appeared as a large colonies (C), the colonies of cells gradually extended in their size with each adjacent ones unified with each other as in six day of culturing (D), the colonies of cells more extended in their size in six day of culturing (E), MSCs on the third day of the third passage revealed a positive reaction for CD44 indicator and blemished with deep brown color of DAB stain (F).

**3.2.Viability and cells count results:** The results of viability of cells after third passage of culturing showed the stained and non-stained cells ratio were 10% and 90% respectively, the cells were adjusted until obtained the concentration 2.5x $10^6$ cell/ml.

### 3.3. Effect of 4-Vinylcyclo-hexene diepoxide on FSH, LH, estrogen, progesterone after 15 days of injection

The results in Table 1 showed the animals group treated with 4-vinylcyclo- hexene diepoxide (positive standard group) revealed a considerable increase ($P$ <0.05) in altitudes of FSH

and LH when contrasted with the negative baseline group, whereas the levels of estrogen and progesterone showed a statistical decline ($P$ <0.05) when compared with negative baseline group.

**Table 1:** Influence of 4-vinylcyclohexene diepoxide (80 mg/kg) B.W on hormones after 15 days of first injection.

| Hormones /Animals | FSH level (U⁄L) | LH level (U⁄L) | Estrogen level (Pg⁄L) | Progeste-rone level (Ng⁄ml) |
|---|---|---|---|---|
| Control group | 8.56 ± 2.57 B | 8.68±2.59 B | 401.6±9.60 A | 22.54±2.57 A |
| 4-vinylcyclohexene diepoxide group | 26.34±2.57 A | 23.36±2.57 A | 302.44±9.47 B | 11.34±2.56 B |
| *LSD | 7.09 | 7.03 | 36.61 | 6.99 |

*Averages with the various letters in similar columns differed statistically ($P$<0.05).

**3.4. Serum FSH, LH, estrogen, proges-terone and Anti-mullerian hormone levels after 30 days of treatment:** The results in Table 2 showed that levels of FSH and LH boosted statistically ($P$ <0.05) in the T2 when compared with T1, T3 and T4. While the FSH and LH in T3 revealed a significant decline ($P$ <0.05) when compared with T4 with no statistical difference ($P$<0.05) as contrasted with T1. Whereas the FSH and LH levels of T4 revealed a significant raise ($P$<0.05) as contrasted with T1 and a statistically declined ($P$<0.05) as compared with T2, the levels of estrogen, progesterone and anti mullerian hormone were decreased statistically ($P$ <0.05) in T2 when compared with T1, T3 and T4. While the levels of estrogen, progesterone and anti mullerian hormone in T3 revealed a significant increment ($P$<0.05), when compared with T4 and T2, with no statistical variation ($P$<0.05) when contrasted with T1. The levels of estrogen, progesterone and anti-mullerian hormone of T4 illustrated a statistical raise ($P$ <0.05) when contrasted with T2, in addition to a significant decrease ($P$<0.05) in estrogen and progesterone levels when compared with T3 and T1.

**Table 2:** Effect of Mesenchymal stem cells 25x $10^6$(cell /kg), Clomiphene Citrate 0.1 mg/kg B.W and Phosphate buffer saline on hormones of mice treated for 30 days.

| Groups | Hormones | | | | |
|---|---|---|---|---|---|
| | FSH (U⁄L) | LH (U⁄L) | E2 (Pg⁄L) | Prog. (Ng⁄ml) | AMH (Ng/ml) |
| T1 | 8.98 ± 2.60 C | 8.56 ± 2.03 C | 401.6 ± 9.60 A | 21.53 ± 3.57 A | 8.01 ± 1.52 A |
| T2 | 25.3 ± 2.80 A | 23.57 ± 3.07 A | 302.44 ± 9.47 C | 10.35 ± 2.47 C | 5.92 ± 1.30 B |
| T3 | 8.40 ± 2.43 C | 9.02 ± 2.08 C | 416.62 ± 6.75 A | 22.36 ± 3.50 A | 7.93 ± 0.95 A |
| T4 | 10.11 ± 2.65 B | 10.3 ±2.02 B | 358.02± 6.67 B | 19.41 ± 3.77 B | 7.25 ± 0.79 A |
| *LSD | 1.01 | 1.09 | 20.13 | 2.12 | 0.91 |

*Mean ± SE *Averages with the various letters in similar columns differed statistically ($P$<0.05).

**3.5. Glutathione peroxides and superoxide dismutase**
The results of glutathione peroxides and superoxide dismutase are demon-strated in Table 3. The altitudes of superoxide dismutase and glutathione peroxides enzymes of T2 and T4 demonstrated a significant decline ($P$<0.05) in comparison with the T1 and T3. While the altitudes of glutathione peroxides and superoxide dismutase enzymes in T3 appeared no significant variation ($P$< 0.05) in comparison with T1.

**Table 3:** Effect of Mesenchymal stem cells 25x $10^6$(cell /kg), Clomiphene Citrate 0.1 mg/kg B.W and Phosphate buffer saline on Glutathione peroxides (GPX) (mmol/mg) and superoxide dismutase (SOD) (u/ml) in serum of mice treated for 30 days**.**

| Enzymes / Groups | Glutathione peroxides (GPX) (mmol/mg) | Superoxide dismutase (SOD) (u/ml) |
|---|---|---|
| T1 | 8.04± 3.07 A | 11.41 ± 2.77 A |
| T2 | 5.06± 2.50 B | 8.22± 2.59 B |
| T3 | 7.27± 2.57 A | 11.57 ± 2.01 A |
| T4 | 5.27 ± 2.59 B | 9.50 ±3.26 B |
| *LSD | 0.92 | 2.41 |

* Mean ± SE *Averages with the various letters in similar columns differed statistically ($P$<0.05).

**3.6. Number of embryos and Fertility index %**
The number of embryos in T3 and T4 appeared a significant increment ($P$< 0.05) as compared with T1, in addition to disappeared the number of embryos and fertility index in T2 as compared with T1, T3 and T4. The intra peritoneal injection of Mesenchymal Stem Cells caused an enhancement in fertility index as compared with T4 with no variation when contrasted with T1. While the fertility index in T4 was decreased when contrasted with T1. as in Table 4.

**Table 4:** Effect of Mesenchymal Stem Cells 25x $10^6$(cell /kg), Clomiphene Citrate 0.1 mg/kg B.W and Phosphate buffer saline on number of embryos and Fertility index% of female mice treated for 30 days.

| Parameter / Groups | Number of embryos | Fertility index% |
|---|---|---|
| T1 | 8.80 ± 1.94 B | 100% |
| T2 | 0 ± 0 | 0% |
| T3 | 11.91 ± 1.86 A | 100% |
| T4 | 10.03 ± 1.99 A | 80% |

* Mean ± SE * LSD for number of embryos= 1.91 *Averages with the various letters in similar columns differed statistically ($P$<0.05).

**3.7. Ovaries weight ratio**
The body weight, ovaries weight and ovary weight ratio in T2 indicated to a statistical decrease ($P$<0.05) when compared with T1, T3 and T4. While the body weight, ovaries weight and ovary weight ratio in T3 and T4 appeared a significant elevation ($P$< 0.05) as contrasted with T1, Table 5.

**Table 5:** Influence of Mesenchymal Stem Cells 25x $10^6$(cell /kg), Clomiphene Citrate 0.1 mg/kg B.W and Phosphate buffer saline on body weight (gm) and ovary weight(gm) and ovary weight ratio (%) of mice treated for 30 days.

| Parameters / Groups | Body weight (gm) | Ovary weight (gm) | Ovary weight ratio (%) |
|---|---|---|---|
| T1 | 30.36 ± 1.33 A | 0.015 ±0.58 B | 0.049 ± 0.51 B |
| T2 | 27.57 ± 1.04 C | 0.011 ±0.36 C | 0.039 ± 0.46 C |
| T3 | 30.00 ± 1.24 A | 0.016 ± 0.30 A | 0.053 ±0.68 A |
| T4 | 29.66 ± 1.68 B | 0.016 ± 0.81 A | 0.053 ± 0.71A |
| LSD | 1.96 | 0.001 | 0.013 |

* Mean ± SE *Averages with the various letters in similar columns differed statistically ($P$<0.05).

### 3.8. Serums Vascular Endothelium Growth Factor.

The serums vascular endothelial growth factor level in T2 revealed a statistical depletion ($P$<0.05) in contrasted with the T1, T3 and T4, whereas the intra peritoneal injection of Mesenchymal Stem Cells to mice showed the best significant raise ($P$ <0.05) in the serums vascular endothelial growth factor levels when compared with T1, T3 and T4. The serum vascular endothelial growth factor level in T4 showed no statistical variation ($P$ <0.05) when contrasted with T1 as in Table 6.

**Table 6:** Influence of Mesenchymal stem cells 25x $10^6$(cell /kg), Clomiphene Citrate 0.1 mg/kg B.W and Phosphate buffer saline on Serum Vascular Endothelial Growth Factor (ng/ml) of mice treated for 30 day.

| Parameter / Groups | Serum Vascular Endothelial Growth Factor (ng/ml) |
|---|---|
| T1 | 51.10 ± 4.90 B |
| T2 | 47.91 ± 5.81 C |
| T3 | 55.34 ± 5.12 A |
| T4 | 50.23 ± 4.75 B |
| LSD | 0.86 |

*Averages with the various letters in similar columns differed statistically ($P$<0.05).

### 3.9. Histopathological and Imuno—histochemistry

After 15 days from first injection of 4- vinylcyclohexene diepoxide (80 mg/kg) B.W to mice, the ovarian tissues were taken from animals in diestrus phase and the histopathological section of tissue showed atretic follicles (AF) with variable degrees of degeneration of secondary follicles together with stoma vascular congestion as in (Fig. 2), while after 30 days the examined ovarian tissues of T2 showed moderate blood vessels dilation and filled with edematous fluid together massive vaculation of stromal cells with multiple atretic follicles (Fig. 3) when compared with normal section of ovarian tissue in T1 (Fig. 7). The histopathological section of ovary from T3 showed the best improvement in ovarian tissue after compared with other treated groups, as in (Fig. 4), the ovarian tissue revealed regular outline of growing follicles mature follicles with focal lymphocytic aggregation that replaced the antral space, in addition in other section, the tissue showed mature follicles with early antrum theca follicle together with evidence of primordial follicles and secondary follicles as in (Fig. 5). Whereas, the histopathological section of ovary from T4 showed widespread edema accompanied with blood vessels congestion and dilation seen through medullar section with evidence of vacuolation of surrounding the follicles as in (Fig. 6). The imunohistochemistry results after thirty day of treatment of the ovarian tissues from T2 revealed a negative immune-staining reaction for CD44 marker in atretic follicles as in (Fig. 8). While the Immunohistochmestry section of ovary tissue from T3 showed very deep strong positive brown reaction for CD44 of both stromal cells and surface epithelium in addition to positive immune-reactivity of primary ovarian follicular cell as in (Fig. 9). Whereas, the ovarian tissues from T4 expressed negative to CD44 marker immune-staining reaction in primary and secondary follicles as in (Fig. 10), the normal untreated mice T1 also showed negative immune-staining reaction in ovarian follicles and its interstitial stromal as in (Fig. 11).

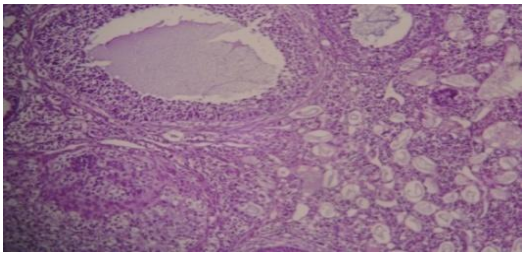

**Fig 2:** The histopathological section of ovary from mice after treatment with 4-vinylcyclohexene diepoxide showed atretic follicles with variable degrees of degeneration of follicles H & E stain, 40x).

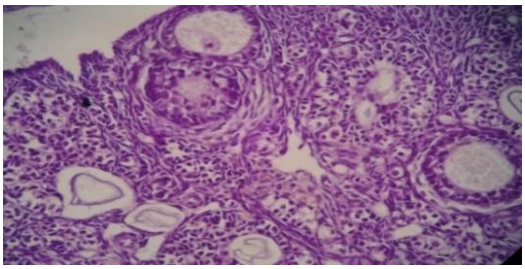

**Fig 3:** The histopathological section of ovary from T2 showed moderate blood vessels dilation and filled with edematous fluid of stromal cells with multiple atretic follicles (H&E stain, 40x).

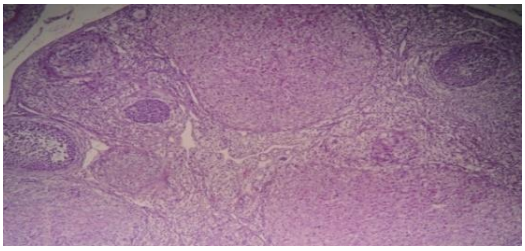

**Fig 4:** The histopathological section of ovary from T3 showed regular outline of growing mature follicles with focal lymphocytic aggregation that replaced the antral space (H&E stain, 40x).

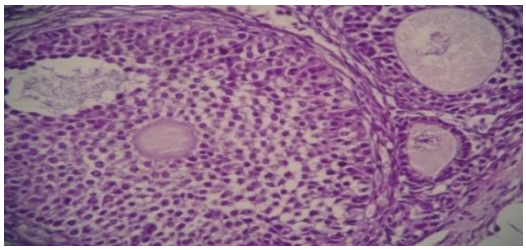

**Fig 5:** The histopathological section of ovary from T3 showed mature follicles with early antrum theca follicle together with evidence of primordial follicles and secondary follicles (H&E stain, 40x).

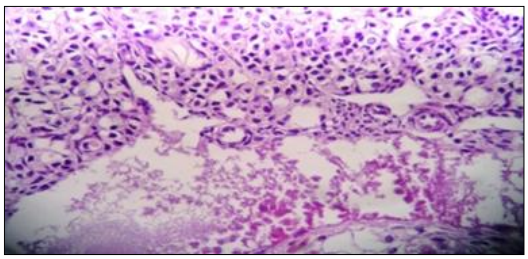

**Fig 6:** The histopathological section of ovary from T4 showed widespread edema accompanied with blood vessels congestion and dilation seen through medullar section with vacuolation of follicles (H&E stain, 40x).

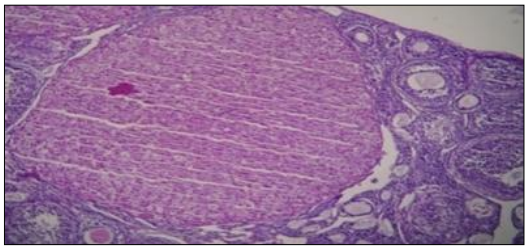

**Fig 7:** The histopathological section of ovary from T1 showed normal cortical and meduller tissue camped of several cortical follicles at different stage of development, C.L and cortical stroma. (H&E stain, 40x).

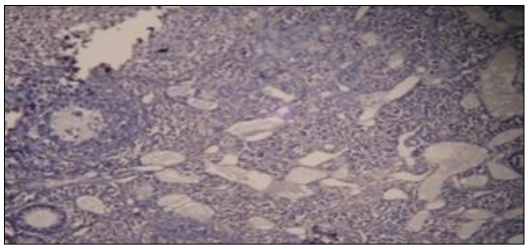

**Fig 8:** The Immunohistochmestry section of ovary tissue from T2 showed negative immune-staining reaction for CD44 marker in primary and secondary follicles including atiral follicles (immunostain, 40x).

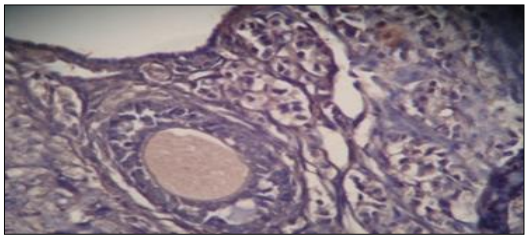

**Fig 9:** The Immunohistochmestry section of ovary tissue from T3 showed very deep strong positive brown reaction for CD44 of both stromal cells and surface epithet-lium in addition to positive immunoreactivity of primary ovarian follicular cell (immunostain, 40x).

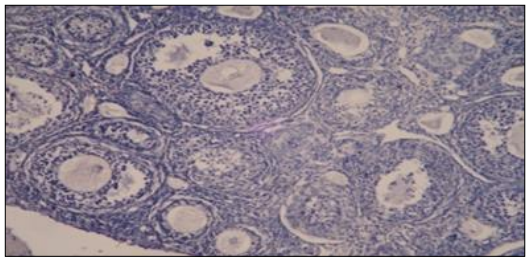

**Fig 10:** The Immunohistochmestry section of ovary tissue from T4 expressed negative to CD44 marker immune-staining reaction in primary & secondary follicles (x40).

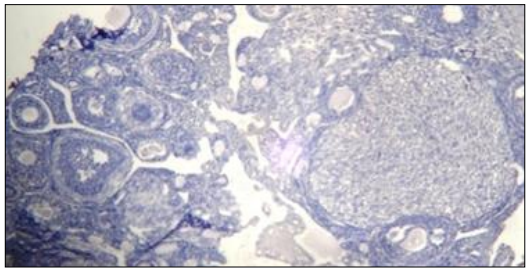

**Fig 11:** The section of ovary tissue in T1 showed negative to CD44 immunostaing reaction in follicles and its interstitial stromal cell (immunostain, 40x).

## 4. Discussion

The using of a culture medium RPMI with 10% FCS lead to a successful MSCs collection from bone marrow, the serum is supplemented to the BM culture as enhancing factors for the stem cells colony growth, in addition the results of culturing in first, second and third passages are corresponded to results described by Jing *et al.*, [15]. who has been noticed that number of floating cells increased during the first day and after feeding process, the basis for this augment may be to get rid of the toxic materials resultant from metabolic processes of cells and due to their action for division and proliferation, these consequences are in conformity with previous research mentioned by Liu *et al.*, [17]. The result of the present study established the capacity of MSCs for proliferation and increasing in number with their ability for attached in the culture flasks, this result perhaps due to the stem cells are divided to give the progenitor cells, these cells are self-replicate as well as, another cell which is committed to final differentiation to certain direction of cells with spindle like shape, this result was in agreement with findings described by previous report [18]. The results of phenotypic analysis of MSCs can be attributed to the ability of Anti-CD44 antibody to identify MSCs indicating to that cells are primarily of Mesenchymal origin through antigen antibody interactions. This observation is in agreement with study recorded by Afifi and OLfat [19]. The increase levels of LH and FSH and decrease levels of progesterone and estrogen in mice group treated with 4-vinylcyclohexene diepoxide after 15 days of the first injection and in T2 after 30 days of treatment, perhaps clearly attributed to be the 4-vinylcyclohexene diepoxide acted as ovotoxic lead to disturbance in hypothalamic-pituitary signaling and increased releasing the oxidative stress in granulose cells lead to damage of ovarian small follicles (primordial follicles) and de-sensitivity of ovarian tissue to FSH and LH and then frequency of hypothalamic gon-adotropin releasing hormone and hypersecretion of LH and FSH in the blood as well as depletion in levels of estrogen and progesterone, in addition the granulose cells are responsible for releasing Anti-Mullerian (AMH) hormone, this may be explained the reasons

of decline in level of (AMH) after given 4-vinylcyclohexene diepoxide to mice, these consequences are in concurrence with results demonstrated by Connie *et al*., [20]. The improvement in hormones levels of FSH, LH, estrogen, progesterone and (AMH) in T3 may be attributed to the Mesenchymal stem cells had the ability for residing and rescuing ovarian function, in addition to inhibiting the stromal cells apoptosis during the excretion of stanniocalcin-1 and several additional paracrine aspects in addition to secrete bioactive molecules that promoted tissue repairing, by regulation of granulose cell apoptosis leading to increase in uptake of FSH hormone by ovarian tissue and regulation in production of AMH hormones which may be played an important role in ovarian folliculogenesis and thus acted as a useful marker of ovarian reserve lead to an increase in the numeral of follicles furthermore, increase in estrogen, progesterone levels, these results supported that previous study recorded by Lee *et al*., [21].Whereas, the FSH and LH levels of mice in T4 was decreased when compared with (PBS) treated group but increased when compared with control group, this result can be clarified by the anti-oestrogen activity of Clomiphene seemed to be associated to the block hypothalamus oestrogen receptors lead to inhibiting negative feedback of estrogen on gonadotropin release resulted in up regulation of the hypothalamus-pituitary-gonads axis, the increase in releasing of pituitary gonadotropin (in particular FSH which specifically acted on the mechanisms of follicle maturation in the ovaries), which stimulated the endocrine activity and maturation of the follicles, lead to enhancement the response of the ovary to uptake the FSH and LH, in addition to the following expansion of the corpus luteum and then maintained dominance of the progesterone levels, furthermore, Clomiphene citrate acted as a stimulating to anti mullerian hormone, this observation was also described by report recorded by Fleeger *et al.,* [22], as well as Clomiphene citrate was played a chief role in follicular regulation, development and steroidogensis by stimulating growth hormones with insulin growth factor-1 (IGF-1), this result was supported by other findings reported by Henawi and Aljahdali [23]. The decreases in altitudes of superoxide dismutase and Glutathione peroxides of mice in T2 may be attributed to the 4-vinylcyclohexene diepoxide (VCD) caused discrim-inating destruction of ovaries follicles via accelerating the normal apoptotic process of atresia. Lead to increase the free radicals, this finding is in agreement with the result reported by Connie *et al*., [20]. While the increment in glutathione peroxides and superoxide dismutase enzymes levels of T3 may be regarded to MSCs had the ability for detoxifying the injuries sites and of hydrogen peroxide lead to accelerated tissue healing [21]. Furthermore, BMSCs had the potential effect to repair ovary structure and could be helped in improving the damaged in niches of ovaries by scavenging the free radicals, the resembling result reported by Fu *et al.* [24] The decreases in altitudes of Glutathione peroxides and superoxide dismutase of mice in T4 perhaps resulted from Clomiphene citrate treatment had the ability in increasing the hydrogen peroxide (H2O2) levels and decreasing the catalase activity [25]. The increase in levels of ROS can be induced overexpression of protein and thereby DNA disintegration both in oocytes and granulosa cells lead to depletion the Glutathione peroxides and superoxide dismutase [26]. The disappearance of embryos number and fertility index in T2 may be due VCD caused selective damage of ovarian small follicles lead to increasing the normal, apoptotic processes of atresia, resulted in disturbance in the hypothalamic-pituitary signaling, then follicle loss and disruption the normal oocyte growth [27]. While the intra peritoneal injection of Mesenchymal stem cells caused an enhancement in number of embryos and fertility index as compared with T2, this result may be regarded to that Mesenchymal stem had the ability in reconstitution of oogenesis and preparation the ova for fertilization by reducing granulose cell apoptosis through effects on its G-protein coupled receptor protein signaling and MAPK pathways, both of which are also important for follicle and oocyte growth, in addition to stimulate the secretion of bioactive mediators that favor cell growth, so the MSCs had a potential effect for repairing injured tissue and also a strong tool in restoring pregnancy and fertility, these results of present study are consistent with prior reports by Augello *et al*., [28]. While the decrease of fertility index in mice treated with Clomiphine citrate as compared with control group may be attributed to Clomiphine citrate induced reduce in level of estradiol 17β lead to deteriorated quality of oocyte by inducing oocytes susceptibility towards apoptosis. The anti-estrogenic effect of Clomiphene citrate treatment is considered one of the main side effects that lead to the maturation, development of oocytes and inhibition in oocyte quality after ovulation which effect on pregnancy rate [29]. In the same time the increased in the number of embryos may be resulted from Clomiphene citrate induced an augment in endogenous FSH discharge and enhanced the uptake of FSH in ovarian tissue by increasing sensitization of ovarian tissue to endogenous FSH that lead to stimulating multi-follicle development [30]. The decrease ovary weight ratio of T2 perhaps is due to the VCD caused reducing in weight gain by depletion the appetite, in addition to, decrement in ovarian weights accrued by lossing of large antral follicles [31]. The elevation ovary weight ratio of T3 may be attributed to MSCs injection lead to enhancement the number of follicles, and serum vascular endothelial growth factor in addition to improved and promoted the renewal of inhabitant cells, this efficacy is most possibly mediated by paracrine effects that are started in response to MSCs excreted cytokines and growth factors that prevented necrosis and cell death, [32]. While the increase ovary weight ratio in T4 as compared with T2 may attributed to Clomiphine citrate increased appetite as well as to fatness and hyperinsulinaemia are well associated with Clomiphene citrate, in addition to increase the number of follicles lead to increase ovary weight, furthermore, Clomiphine citrate played an important role in enhancing growth factor and IGF-1, this result supported by Parsanezhad *et al*., [33]. The depletion in serum vascular endothelial growth factor level of mice in T2 may be resulted from 4-vinylcyclohexene diepoxide had the ability in accelerating the natural apoptotic process of atresia, by inhibiting autophosphorylation of c-kit receptor, that is considered as a key molecule in a serious signals pathway linked with ovarian cell development, and therefore disturbed the typical oocyte growth leading to depletion in SVEGF, these results correspond to different studies such as [34]. While the intra peritoneal injection of Mesenchymal Stem Cells to mice showed the best raise in the serum vascular endothelial growth factor levels, this result may be attributed to MSCs secreted bioactive factors that affect ovarian regeneration and restore, MSCs had paracrine effects lead to secretion of VEGF that stops cell apoptosis, increments the tissues vascularity and supporters regeneration of tissues, in addition to secretion growth factors and cytokines that prevented necrosis and programmed cell death, this result of present study was consistent with prior report by Somia *et al.*, [35].

Whereas, the increase serum vascular endothelial growth factor level in T4 as compared with phosphate buffer saline treated group can be clarified by Clomiphine cirate had the ability in increasing of IGF and growth hormones which stimulated the tissues and cell growth lead to enhancing the secretion of the growth factor from endothelium [23]. The ovarian tissues changes after 15 and 30 days, was possibly due to ovotoxic effects of VCD seem to firstly directly target small follicles, VCD caused lakes of small follicles, elevated serum FSH, and cyclic disturbance foregoing impulsive ovarian aging that was identical to occasions that occurred through the start of menopause in animals [36]. VCD can be accelerated apoptotic process of atresia, and inhibited autophosphorylation of c-kit receptor [34]. While the histopathological changes in the ovarian tissues of T3 may be attributed to the Mesenchymal stem cells (MSCs) had the ability to restore ovaries dysfunction by either engrafted to damage tissue or secreted bioactive molecules, this result agreed with result reported by Ranganath *et al*., [37]. In addition, to the paracrine effects that are started in response to MSCs excreted cytokines and growth factors like vascular endothelial growth factor that prevented necrosis and cell death, as well as can be inhibited follicular cell atresia and follicles apoptosis and can be improved ovarian function and structure damage, the same result reported by Molloy *et al*., [32]. The histopathological changes of ovary from T4 may be explained by Clomiphene citrate increased the levels of free radicals lead to DNA disintegration both in granulosa cells and oocytes and congestion of blood vessels, as well as Clomiphene citrate increase uptake of granulose cell to FSH and LH lead to enlargement of ovaries with multiple follicles, the similar results reported by Tripathi *et al.,* [26]. While the Immunohistochemistry section of ovary tissue from T3 showed very deep strong positive brown reaction for CD44 of both stromal cells and surface epithelium as compared with other treated groups, this result may be regarded to the incorporation of MSCs into the tissues of ovaries with alteration of dented cells and the detection of endogenous MSCs by using CD44 immuno-reactivity marker through a specific antigen/antibody reaction with applied the labeled avidin–biotinperoxidase, which linked to the biotin on the secondary antibody, the position of antibody binding was visualized by diaminobenzedine chromogen, which is turned into a brown impulsive by peroxidase which is specialized for detecting MSCs and then the CD44-positive cells was appeared as brown cytoplasmic deposits, this result in agreement with study recorded by Gosden, [38].

## 5. Conclusion

Mesenchymal stem cells at dose (25x $10^6$cell /kg) lead to more protection effect on fertility, hormones, antioxidant enzymes, serum vascular endothelial growth factor and ovarian tissue than Clomiphine citrate (0.1 mg/kg) against the toxic effect of 4-vinylcyclohexene diepoxide.

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