Transdifferentiation of Bone Marrow derived mesenchymal stem cells into oocytes a step towords restoration of ovarian activity in cases of premature ovarian failure

Prof. Dr. Asmaa Fath Elbab; Prof. Dr. Hala Gabr; Dr. Eman Elsherif

Obstetrics and Gynecology Department, Faculty of Medicine (Girls) Al-Azhar University, Egypt,
Clinical Pathology Department, Faculty of Medicine, Cairo University, Egypt,
MB. Bch, Msc, Obstetrics and Gynecology Department Specialist, Ministry of Health, Egypt

Abstract: Background: The majority of cases with premature ovarian failure (POF) are not concerned about the lack of menstrual cycles or ovarian steroids, but about virtually no chance of conceiving with genetically related children. Although oocyte or embryo donation is an alternative method for achieving a pregnancy, yet it is religiously not allowed in some countries and the majority of cases prefer to reproduce with their own gametes. Stem cell therapy is a rapidly evolving field that could help the recovery of ovarian function in some women who suffered POF. This study aims is to assess the therapeutic potency of bone marrow MSC transplantation for primary and chemotherapy-induced ovarian damage with restoration of ovarian function, and to asses if BM MSC can exert a protective action against onset and severity of chemotherapy induced ovarian damage if given simultaneously with chemotherapy. **Method:** 54 mature white albino female rats were included in the study divided randomly in to 4 groups control (n=9), groups A (n=15), B (n=14), and c (n=16) where ovarian failure was induced by intraperitoneal injection of cyclophosphamide 50mg/kg as a loading dose then 8mg /kg daily dose for 14 consecutive days. Group C injected intaperitoneally with BM MSC at the time of giving chemotherapy to assess if this has a protective action, while group B was injected with BM MSC after confirmation of ovarian failure then followed for a period of 3 weeks before being sacrificed**.** Also 25 patients diagnosed with idiopathic POF were enrolled in the study. Laparoscopic intraovarian injection of autologous BM MSC was performed, patients followed for a period ranging between 8 to 12 months. **Results:** Treated animals showed increased levels of the sex hormone E2, with homing of the iron tagged stem cells in the ovaries confirmed by histopathology. Some human patients showed initial improvement in the form of initial decline of serum levels of FSH with rise of E2 and a tiny increase of AMH. 11 patients of 25 (44%) had resumed their menses at different points of follow up one case had spontaneous successful term pregnancy. **Conclusion:** The current study supports the existence of stem cells in adult mammalian female's bone marrow that can influence ovarian function, even though in this study BM stem cell transplantation did not yield mature eggs and the improved ovarian function together with resumption of menstrual cycle was transient before relapsing again but this moves us a step closer to the desirable goal. **Recommendations:** Understanding the complexity of recovery of ovarian function in POF after stem cell transplantation, cause of ovarian failure whether spontaneous or related to chemotherapy and how ovarian stem cells interact with their surrounding environment with determination of the MSC dose according to its biological effectiveness is a critical point for achieving a successful cell based therapy.

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Key words: Premature ovarian failure (PO F), Chemotherapy induced ovarian failure, Bone marrow derived mesenchymal stem cells (BM MSC)

1. Introduction

Increasing attention is being paid to premature ovarian failure (POF), which is one of the most threatening diseases to female reproductive health. Approximately 1-5% of women worldwide experience cessation of their menstrual cycle prior to age of 40, a condition known as POF. **[1]** ***(Rebar RW, et al, 2008).***

Advances in the treatment of childhood cancer have resulted in markedly improved survival rates. However, with these advancements, cancer survivors now face the long-term consequences of treatment with intensive, multimodality therapies. **[2]** (***Bandyopadhyay S, et al, 2006).***

Given the limited treatment options for women with POI, treatment of POI is performed with two propose: the **first** being hormone replacement therapy (HRT) to reduce complications due to impaired endocrine function of ovaries, However, HRT has been shown to increase the risk of breast cancer **[3]** ***(Antoine C, et al, 2014)*** and the **second** for fertility concerns. Infertility treatments available for POI which may be used before or during ovarian failure, especially in cancer patients, include fertility

preservation such as ovarian cortex, oocyte and embryo cryopreservation, oocyte or embryo donation and adoption in women without any ovarian function.[4] ***(Bl umenfeld Z.et al, 2011).***

Stem cell transplantation has become a promising tool in rescuing damaged ovarian function. Its therapeutic potential has opened up a new way for preserving or recovering the damaged fertility of women receiving chemotherapy. **[5] (**Oxford Journals, Volume 2005).

The application of stem cells transplantation is often associated with controversies, such as safety, source, and their poor survival time *in vivo*. **[6] (*Kestendjieva S. et al, 2008).***

The bone marrow stem cell is the best-studied transplanted stem cell, which contains multiple cell types, such as mesenchymal stem cells (MSCs) and hematopoietic stem cells**. [7] *(Ohishi M., et al, 2010)*** Recent studies suggest that bone marrow stem cells (BMSCs) are promising grafts to treat a variety of diseases, including reproductive dysfunction**. [8] *(Charles Sklar, et al, 2005).***

Method

This study was a prospective animal study and a pilot human study in collaboration between Al-Azhar university (the Obstetrics and Gynecology Department' Alzahraa university Hospital), and Cairo university (Histopathology Department and the Animal Biology & Reproduction unit), Cairo, Egypt.

A total starting number of 63 mature white albino female rats were included in the study, this number was reduced to 54 after injection of chemotherapy as some rats passed away after CTX (9 rats). Their age at the beginning of the experiment was 60-70 days and their weight range was 200-250 mg. rats were fed by rat chow with free access to water, under controlled temperature (28-30C) and relative humidity (50-60%). They were observed for 14 days prior to commencing treatment to ensure adequate adaptation.

Also 25 patients diagnosed with idiopathic POF were enrolled in the study according to ethical committee rules of Al-Azhar university and after verbal and written consents were taken.

I- Animal procedures

Induction of ovarian failure

The adult female rats were randomly divided into 2 groups: control group 9 rats and study group 54 rats. The study group was randomly subdivided into 3 groups before injecting chemotherapy (groups A, B and C respectively) each group consists of 18 rats. Blood samples were collected from each group for determination of baseline levels of estradiol (E_2) and follicular stimulating hormone (FSH). Induction of ovarian failure by Chemotherapy was done by intraperitoneal (IP) injection of cyclophosphamide 50mg/kg as a loading dose then 8mg /kg daily dose for 14 consecutive days. Groups A & B given chemotherapy only. group C received intraperitoneal single dose of stem cells [5x 10^6 cells/kg] from the start at the same time of injecting chemotherapy to evaluate its protective action against onset of chemotherapeutic induced ovarian damage. 9 rats of the study group passed away in the early few days after administration of CTX, 3 rats from group A, 4 rats from group B and 2 rats of group C. So, the number of the study group reduced to 45 rats (15rats, 14 rats, and 16 rats) for groups A, B, and C respectively. After 2 weeks of injecting chemotherapy, ovarian failure was confirmed by measuring serum FSH & E2 and histopathological examination of the ovaries of 2 scarified rats from each group. After confirming ovarian failure, group B was intraperitoneally injected with bone marrow mesenchymal stem cells in a dose of 5x10^6 MSC/Kg in 20 µL saline. Follow up of E2 & FSH weekly, then histopathological examination of the ovaries after scarifying these animals was done to evaluate their folliculogenesis. This was achieved after 4 weeks of stem cell injection.

Preparation of rat Bone Marrow-derived MSC

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old white albino rats with Gibco- BRL DMEM medium (Dulbecco's Modified Eagle's Medium, from Gibco. Mononuclear cells were isolated using ficol hypaque density gradient centrifugation and re-suspended in complete culture medium supplemented with 1% penicillin-streptomycin. Cells were incubated at 37[degrees] C in 5% humidified CO2 for 12-14 days as primary culture with biweekly refreshment of media until 80-90% confluence is reached. Then cultures were washed twice with PBS (Phosphate buffered saline) and the cells were trypsinized with 0.25% trypsin in 1mm EDTA (Gibco-BRL) for 5 min at 37[degrees] C, centrifuged, and cells were tagged by iron oxide particle and prepared for injection by suspension in sterile saline. MSCs identification was evaluated by morphology and flowcytometry. MSC homing was evaluated by Prussian Blue stain for iron oxide tagged cells.

Injection of stem cells

The study group A (15 rats) didn't receive stem cells, 2 subgroups of the study group (B & C) received intraperitoneal single dose of stem cells (5x10^6 MSC/Kg in 20 µL saline). Group B (14 rats) received stem cells after 2 weeks of chemotherapy and after confirming ovarian failure, group C (16 rats) received stem cells at the same time of chemotherapy to check if stem cell transplantation has protective action against onset of ovarian failure if given simultaneously with chemotherapy.

Follow-up

- **Hormonal assay**

This was carried out by weekly estimation of serum E2 and FSH after injections of CTX to assess the changes in ovarian function. Blood samples were obtained from rats by retro-orbital puncture. Samples were collected, transferred directly to the lab then incubated at room temperature for 1 h, and centrifuged to separate the supernatant. The concentration of the hormones was determined by ELISA kits (Enzyme Linked Immuno- sorbent Assay).

Tracking BMSCs by immunohistochemical staining

Rats were sacrificed 3 rats from each group randomly at the end of the study to observe the resurrection of ovarian activity (follicular growth and count) by histopathological examination and tracking of the iron chelated stem cells.

II- Human pilot study

Patient selection:

Of 60 patient, 25 patients fullfilled the inclusion and exclusion criteria and were included in the study.

Inclusion Criteria:

- Age between 18 - 40 years old.
- Patients with normal karyotype spontaneous premature ovarian failure.
- FSH level above (≥ 30 IU/L) repeated twice >4 weeks apart.

Exclusion Criteria:

Abnormal karyotype.

Previous pelvic or abdominal radiotherapy.

Previous surgical management of ovarian pathology.

Chronic disease: renal, liver, cardiac, malignancy.

Past history of infections, endocrine or autoimmune diseases.

Aspiration and preparation of human Bone Marrow-derived MSC

60 ml of bone marrow were aspirated from the posterior iliac crest under local anesthesia and under strict aseptic conditions, 7 days before the assigned time for transplantation. The sample was put in sterile container with appropriate amount of heparin then filtered to remove bone spicules, fat, and cellular debris then processed.

Mononuclear cell separation was done using ficol hypaque density gradient centrifugation. Mononuclear cells were cultured in sterile T25 flasks in complete medium consisting of DMEM, 20% autologous serum, 10% penicillin/streptomycin. Cultures were incubated in humid CO2 incubator at 37 C for 7 days. Cultures medium was refreshed every other day and the morphology was evaluated using inverted microscope. When cells reached 80% confluence, MSCs were harvested by trypsinization for 5 minutes at 37 C. Harvested cells were counted, subjected to viability testing and immunophenotyping using flow cytometry for CD 34, CD 45, CD 90, CD 105 and CD 271. Blood sample drawn from venous blood was centrifuged and then platelets rich plasma (PRP) collected, then purified and finally added to the cells and injected into ovarian tissues.

Laparoscopy and Bone Marrow-derived MSC injection

After giving informed consent, patients were prepared for laparoscopy. BM MSC transplantation was conducted laparoscopically in 21 patients, the other 4 cases were transplanted transvaginally in to the ovarian tissue guided by transvaginal ultrasound, (this was due to some technical obstacles in the operative theatre, and stem cells have to be transplanted within 4- 6 hs if it is in room temperature). Stem cells were transferred directly from the lab to the theatre cooled in ice. Diagnostic laparoscopy allowed for assessment of pelvic anatomy; then stem cells of a dose some peritoneal leakage. There were no complications, and the hospitalization time did not exceed 24 h. patient were then followed by hormonal assessment of FSH, LH, E2, AMH, and ultrasound, antral follicular count at intervals of 2 months for a period of 8-12 months. 3-5 Million Autologous BM MSCs (3ml sample for each ovary) were injected bilaterally in the stroma of both ovaries of the POF patients, using ovum retrieval needles.

After several months of follow up, 2 patients offered by themselves to try another transplantation procedure, especially after the initial improvement of their hormone levels and return of their menstrual cycle. Other patients were counseled to have the same chance, but refused especially it is an invasive surgical procedure carrying physical, psychic stresses in addition to the financial cost.

Statistical methodology

The data collected were tabulated & analyzed by SPSS (statistical package for the social science software) statistical package version 22 on IBM compatible computer.

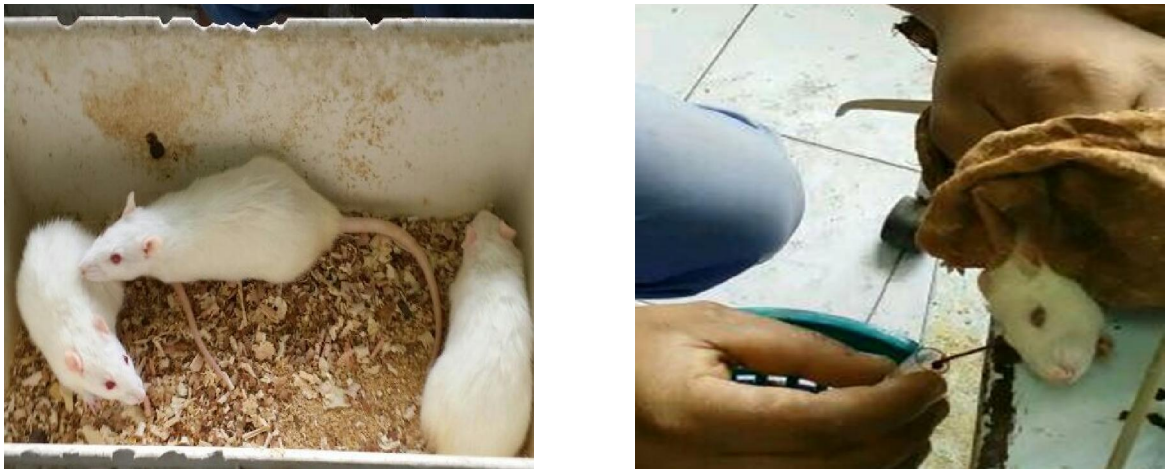
3- Student t- test was done for normally distributed quantitative variables to measure mean and standard deviation and p-value < 0.05 was considered significant.

4- ANOVA test was done to compare three variables**;** one qualitative variable and the other two are quantitative variables of normally distributed variables and p-value < 0.05 was considered significant to detect mean and standard deviation where post hoc tests done to detect the relationship between variables within groups.

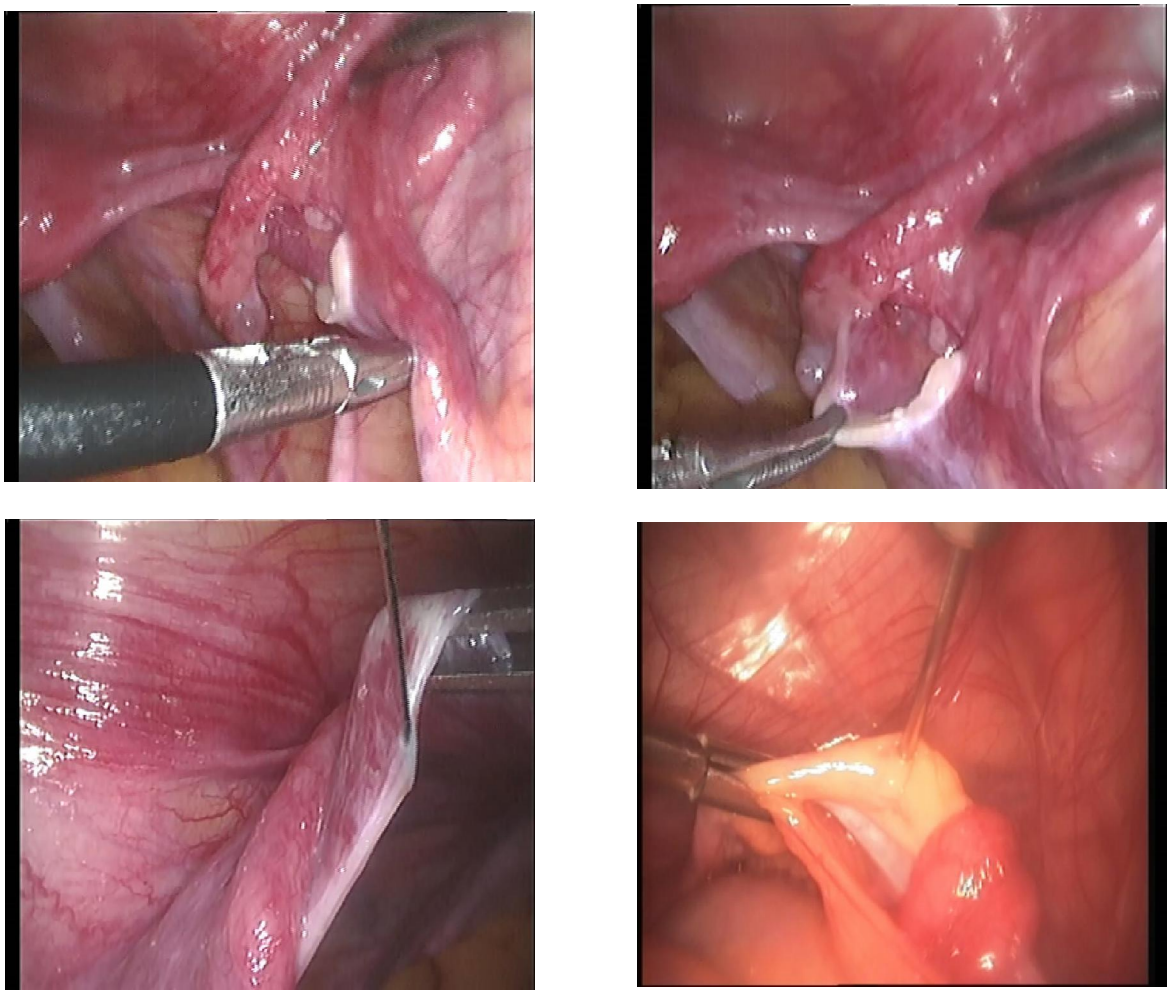
5- LSD test is a post hoc test it was done to variables of significant difference of more than two

groups of normally distributed data after ANOVA test to detect the significant difference between either groups.

All these tests were used as tests of significance at P<0.05,> 0.05 non-significant, <0.01 highly significant. ***[9]*** *(Morton et al., 2001).*



Rats and collection of retroorbital blood samples.



Streak gonads and hypoplastic luteinized ovaries during laparoscopy

Figure 1: E2 levels after stem cell transplantation in rats.

3. Results

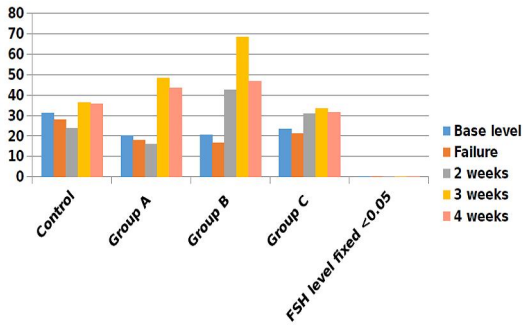
1- Animal results

The starting total number was 65 rats reduced to 54 rats after administration of chemotherapy as some animals passed away after cyclophoshamide. Control group is 9, other groups were 15, 14, and 16 for groups A, B and C respectively.2 groups didn't receive stem cells (control and group A). The other 2 groups received stem cells, group B received stem cells after confirmation of ovarian failure while group C received it at the same time with stem cells.

Table 1 shows E2 (pg/ml) serum concentration in 4 groups in relation to weeks of follow up after chemotherapy and stem cell transplantation. Data are shown as mean± Std. Control group showed mild fluctuation of E2 levels over weeks of follow up. The study groups A, B, and C showed declined levels of E2 after 2 weeks of chemotherapy, this confirmed occurrence of ovarian failure. **Group A** (Chemotherapy only) that didn't receive stem cells then showed slight spontaneous recovery of E2 levels after 3 and 4 weeks of failure. **Group B** that was treated with stem cells after confirmation of ovarian failure showed marked improvement of E2 levels after 2 and 3 weeks of stem cell transplantation, maximum after 3 weeks of stem cells, but declines again after 4 weeks of transplantation. **Group C** that received stem cells from the start and at the same time of CTX showed that E2 levels didn't fall to the degree when compared with the other 2 study groups A & B during period of failure induction, also there was improved E2 after 2 and 3 weeks when compared with base line value, also. This also suggests that BM MSC transplantation on administration of CTX may have protective action against incidence and severity of ovarian damage by chemotherapy. Regarding FSH levels, it didn't show any change over the period of follow up in all groups and remained fixed at a level of <0.05. P-values for groups **B** & **C** can be considered highly statistically significant, because there is statistical difference between means of E2 levels during the follow up period. This means that stem cell transplantation improves the serum level of E2 markedly in group B that was treated with stem cells, and it has protective action in group **C**.

Table 1: serum concentration of E_2 (pg/mL) in the four groups

E2	**Base level of E2**	**After 2 weeks**	**After 4 weeks**	**After 7 weeks**	**After 8 weeks**	**F. test**	**P. value**
Control (n=9)	31.00± 6.20	27.89± 5.16	23.67± 7.31	36.33± 12.50	35.67± 16.71	26.08	<0.001
Group A (n=15) Chemotherapy only	**Base level of E2 before injecting CTX**	**2 weeks after CTX**	**4 weeks after CTX**	**7 weeks after CTX**	**8 weeks after CTX**	**F. test**	**P. value**
	20.00± 3.64	17.73± 2.43	16.00± 3.11	48.40± 22.68	43.60± 18.50	14.4	<0.001
	Base level of E2 before injecting CTX	**Confirmed ovarian failure 2wks after CTX**	**2 weeks after stem cells**	**3 weeks after stem cells**	**4 weeks after stem cells**	**F. test**	**P. value**
Group B (n=14) Stem cells 2 weeks after chemotherapy	20.43±1.99	16.64±1.6	42.40±19.51	68.43±10.92	46.71±12.95	12.0	<0.001
Group C (n=16) Stem cells +chemotherapy together	23.38±5.08	21.19±3.58	30.63±10.17	33.56± 6.31	31.50± 5.80	16.3	<0.001



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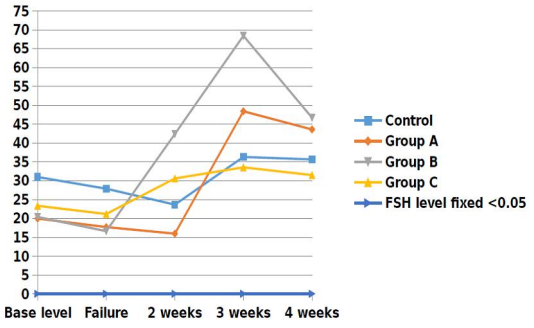

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Figure 1: E2 levels after stem cell transplantation in rats.

Group B that received stem cells 2 weeks after chemotherapy showed marked increase of E2 levels maximally after 3 weeks of stem cell transplantation when compared with groups A (chemotherapy only) and group C that received stem cells from the start together with stem cells. Also in group C that received stem cells from the start, E2 levels didn't reach such lowered levels as in group A and B after chemotherapy.

Characterization and labeling of cultured MSC

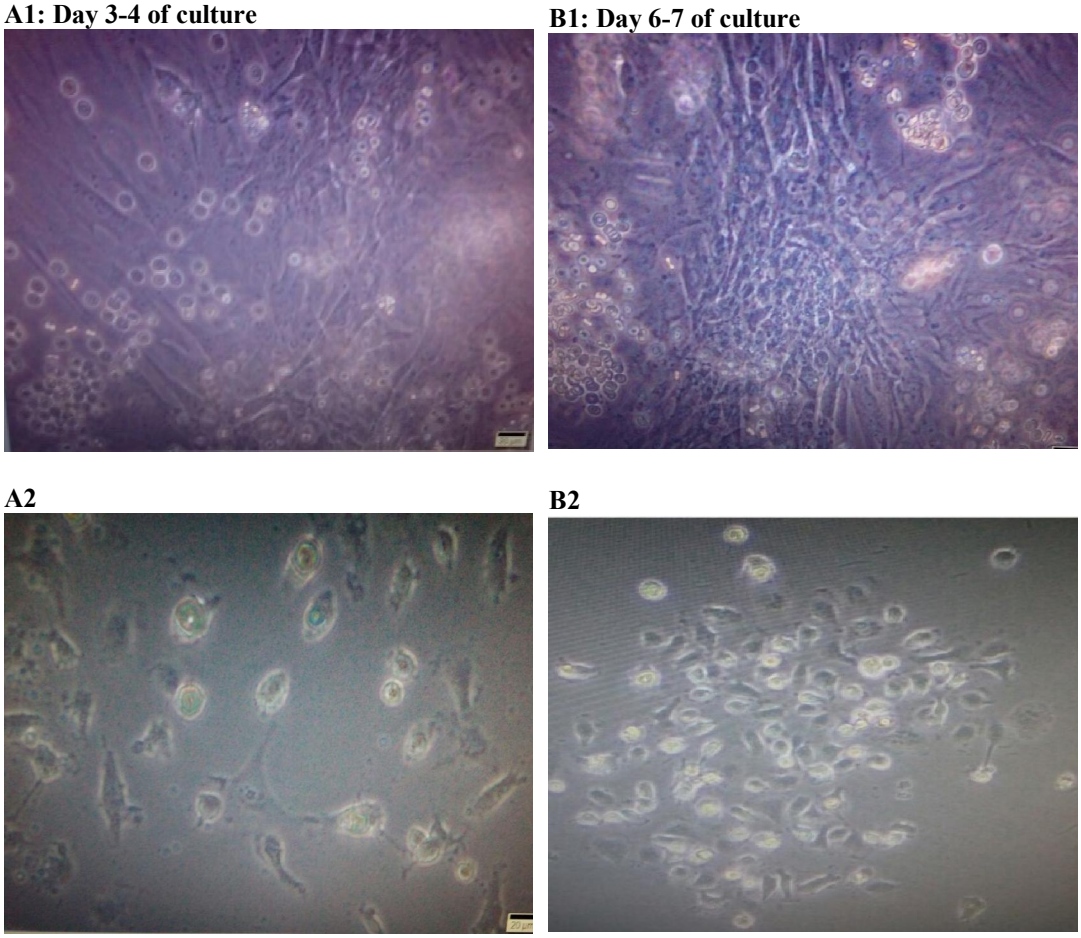

Figure 2: MSC in culture were adherent and morphologically resembled fibroblasts.

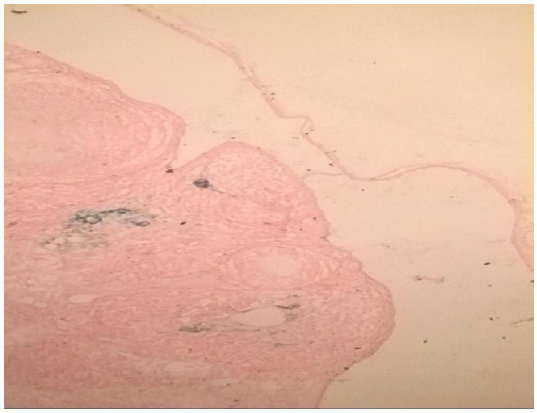

Figure 3: Histology of ovary after stem cell transplantation showing iron positivity due to iron – tagged BM MSC.

MSC tracking *in vivo*

Morphological evaluation of MSC before injection using inverted microscope showing the fibroblastoid morphology of stem cells. **A1** (Low power 40- X) & **A2** (High power 20-x) pictures were taken after 3-4 days of culture. **B1** & **B2** (Low & high power respectively) taken 6-7 days after culture showing increased cell number and density. Histopathological examination and tracking of the iron chelated stem cells.

2-Pilot study

In total 25 patients with POF fulfilled the inclusion criteria and were enrolled in the trial. Autologous BM MSC transplantation was done then patients were followed up at 2 months intervals for a

period ranging between (6-12) months. BM MSC transplantation was conducted laparoscopically in 21 patients, the other 4 cases were transplanted transvaginally in to the ovarian tissue guided by transvaginal ultrasound, (this was due to some technical obstacles in the operative theatre and stem cells have to be transplanted within 4- 6 hs if in room temperature). No other medications received during the period of follow up.

Table 2: Demographics of patients

	Total No	frequency	(%)	Mean	SD Deviation	SD. Error Mean
Age at transplantation	25	25	100	29.2	6.56	1.31
Menarche	25	20	80	10.6	5.45	1.09
Menstrual irregularity	25	9	36	1.6111	0.49	0.097
1ry amenorrhea	25	5	20	1.8	0.41	0.081
2ry amenorrhea	25	17	68	1.16	0.55	0.11
Years of 2ry amenorrhea	25	17	68	1.84	1.98	0.39
1ry infertility	25	4	20	1.24	0.44	0.087
infertility2ry	25	19	76	1.84	0.37	0.075
Endocrine disease	25	0	0%	0	0.0000	0.0000
Autoimmune disease	25	0	0%	0	0.0000	0.0000
Radio or chemotherapy	25	0	0%	0	0.0000	0.0000
Infection	25	0	0%	0	0.00000	0.0000
Pelvic surgery	25	2	8%	1.8889	.32338	0.07622
Family history	25	1	4%	1.9444	0.23570	0.05556

The total number of patients was 18, mean age at transplantation (29.38) years, minimum age was 18 and maximum age was 39 years. mean age of menarche (11) years. 3 patients of 18 have 1ry amenorrhea representing (16.7%), the remaining 15 patients (83.3%) have 2ry amenorrhea with 7 (38.9%) of them have their menses irregular since menarche and mean duration of 2ry amenorrhea (2.14) years. 13 patients (72.2%) of total presented clinically with 1ry infertility, 3 patients presented with 2ry infertility and 2 patients were single presented with 1ry amenorrhea. Two patients (11.1%) had past history of pelvic surgery, one of them was previous ectopic with unilateral salpingectomy, and the other one had ovarian cystectomy. One patient has family history of her mother had early menopause. All patients were – ve for past history of infections, endocrine or autoimmune diseases, and for radio or chemotherapy.

Table 3: Frequency table for clinical data, ultrasonic, laparoscopic findings, interventions done, and outcome

	No	**Percent (%)**
Menstrual irregularity	9	25 %
1ry amenorrhea	5	20 %
2ry Amenorrhea	17	68 %
Still have menses	3	12 %
1ry Infertility	19	76 %
2ry Infertility	4	16 %
Family history	1	4 %
Pelvic surgery	2	8 %
Normal Ut / Cx ratio by US	14	56 %
Hypoplastic uterus by US	5	2 %
Ovaries not seen by US	6	24 %
Hypoplastic ovaries by laparoscopy	12	48 %
Streak gonads by laparoscopy	7	28 %
Interventions		
Laparoscopic injection of stem cells	19	76 %
Vaginal injection of stem cells	6	24 %
Repeated transplant	2	8 %
Outcome		
Improved hormones	17	68 %
Onset of pregnancy	1	4 %
Return of menses	11	44 %

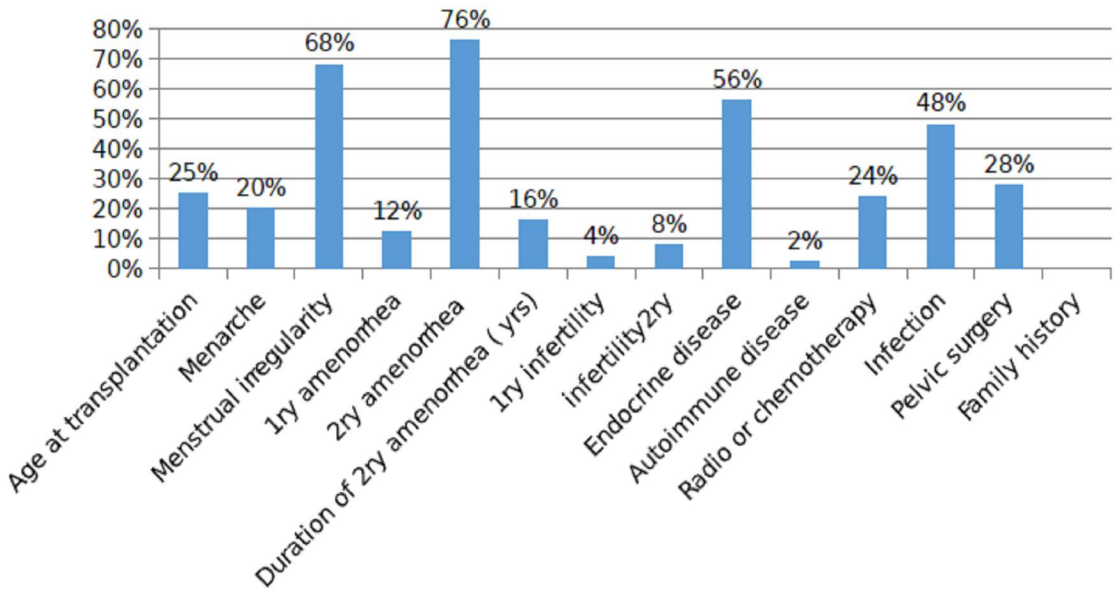

Figure 4: percentage of clinical data presented by patient**.**

Table 4 shows the significance of results of comparison between parameters used for assessment of 25 patients with POF after autologous BMMSC transplantation over a period of 10 months follow up and at 2months intervals. F. test (one way ANOVA test) and p- value used to help determining the significance of the results.

P-value < 0.05 was considered to be statistically significant**.** So, by looking the table up it can be noticed that P-value for FSH, E2 (0.846), A.F.C (0.135), and Endometrial thickness (0.136) all are statically non-significant being larger than 0.05 and this implies that there is no detectable difference of these parameters for the sample size used over the period of follow up.

The P-value for both LH and AMH (< 0.001) is considered statistically highly significant. This actually means that BM MSC actually improved AMH results, so we fail to totally reject this hypothesis.

Figure 5: levels of Sex hormones, AFC and endometrial thickness at 0, 2, 4, 6 a 8 and 10 months after transplantation.

FSH, LH levels showed initial decline after 2 months of BM MSC transplantation before relapsing again, E2 levels markedly increased also after 2 months, then started to decrease gradually then after. AMH, AFC and endometrial thickness levels remained stationary low during 1st 6 months, and it showed a noticeable increase after 8 months.

Table 4: Serum concentrations of Sex hormones, AFC and endometrial thickness. Data are shown as Mean ± SD

Months mean±SD	0	2	4	6	8	10	F.test	p.value
FSH	66.4296 ±30.5021	47.228 ±30.53181	62.2528 ± 29.31872	66.7000 ± 31.32252	72.8000 ±9.54987	72.8000 ±9.5497	2.322	0.082
LH	27.3720 ± 9.77708	23.6640 ± 13.86563	27.0952 ± 11.44750	33.760 ± 15.25964	83.6800 ±20.90184	83.6800 ±20.90184	26.44	0.000
E2	41.3960 ± 31.57563	66.2720 ± 41.85454	63.1160 ± 43.21873	59.1200 ± 46.08626	45.323 ±29.8098	54.120 ±45.675	0.168	0.846
AMH	0.1696 ± 0.13192	0.1416 ± 0.06799	0.1712 ±0.14681	0.2040 ± 0.19891	0.3800 ± 0.17889	0.3800 ±0.1788	3.728	0.015
A.F.C	1.0000 ±.1.457	2.8000 ± 1.97906	3.1200 ± 2.14709	3.1200 ± 2.16641	5.2000 ±0.83666	5.2000 ±.83666	1.909	0.135
End. thickness	4.2800 ± 0.93630	4.5600 ± 1.00333	5.1600 ± 1.31276	5.0400 ± 1.36870	5.8000 ± 1.09545	5.8000 ±1.09545	1.905	0.136

Table 5: levels of FSH, E2, and AMH for the 2 patients over a period of 4 months after a second transplant.

MONTHS AFTER2ND TRANSPLANT Mean ± SD	2	4	P –value
FSH	74.50±30.41	47.228 ±30.53181	0.077
E2	40.0000±8.48528	59.1200± 46.08626	0.092
AMH	0.3000±28284	0.3800 ± 0.17889	0.487

Table 6: comparison of results of hormones, AFC, and endometrial thickness between laparoscopic & transvavinal routes for stem cells injection over months of follow up.

	Laproscopic	vaginal	p.value
FSH 2	69.81±31.2	72.43±30.6	0.94
FSH 4	66.2±25.58	54.10±31.24	0.45
FSH 6	71.78±29.04	63.85±40.22	0.67
E2.2	57.21±37.33	82.00±52.31	0.31
E2.4	54.14±38.64	91.3±37.91	0.21
E2.6	49.81±37.9	89.1±54.72	0.15
AMH	0.17±0.13	0.10±0.00	0.34
AMH.2	0.12±0.05	0.17±0.10	0.25
AMH.4	0.17±0.16	0.15±0.10	0.85
AMH.6	0.19±0.22	0.20±0.20	0.96
A.F.C.2	2.67±1.83	4.00±1.41	0.18
A.F.C.4	2.93±2.13	4.35±0.96	0.246
A.F.C.6	3.00±2.11	4.11±1.41	0.388
End. Thickness 2	4.36±1.01	5.50±0.58	0.47
End. Thickness 4	5.00±1.24	6.10±0.82	0.16
End. Thickness 6	4.82±1.13	5.85±1.71	0.22

As shown in table 6, p-values for all parameters compared over 2, 4, 6 months after transplantation are all more than 0.05 and not statistically significant. so there is no detectable statistical difference between the 2 routes of transplantation.

Outcomes

- **1st outcome: Hormonal levels**

All cases of 1ry amenorrhea (5 cases) did not show any significant difference in the hormonal levels of FSH, E2 or AMH after transplantation. Cases of 2ry amenorrhea that were transplanted either laparoscopically or vaginally, showed initial decline of serum levels of FSH and rise of E2. Few patients showed minimal increase of AMH, 4 patients after 4 months increased to 8 patients after 6 months of transplant. But after 6 months only 2 patients showed continued but very minimal increase of AMH, the maximum AMH level reached was 0.9 raised from 0.2 before transplant.

Table 7: Fisher LSD test

Sig.P-value Pairs	FSH	E2	AMH	A F C
P1 (2-4 mon.)	0.077	0.799	0.487	0.583
P2 (2-6 mon.)	0.023	0.565	0.145	0.583
P3 (2-8 mon.)	0.083	0.348	0.002	0.019
P4 (4-6 mon.)	0.598	0.749	0.441	1.00
P5 (4-8 mon.)	0.470	0.279	.0006	0.042
P6 (6-8 mon.)	0.676	0.206	0.019	0.042

LSD test or the Least Significant Difference used to enables direct comparisons between two individual groups in pairs. p- values for FSH, are non-significant, only one result was significant (P2: 0.023) that compare results of FSH after 2 months with that after 6 months of transplant. P values for E2 were non-significant throughout follow up. For AMH and AFC, p values were significant when comparing levels after 2 months to that after 8 months (P3) and when comparing AMH levels after 4 & 6 months to that after 8 months of transplant.

- **2nd outcome: Return of menses**

From the table below that shows the number and percent (%) of cases that had their menses back, of

total 25 patients 11 had their menses back representing (44 %), all were cases of 2ry amenorrhea but unfortunately this was a transient return with maximum No of cycles 9 irregular cycles for 1 year after transplantation and a minimum of one cycle with 2 patients. This was associated initially with a relatively lowered FSH levels and increased E2 serum levels before starting to relapse again. Results during the first 4 months of follow up were very promising and cannot be ignored. Soon after, almost all cases that showed initial improvement started to relapse again when the FSH levels started to rise, E2 levels dropped back and menses stopped again.

Table 8: 1st Return of menses

	Months after transplantation	No of cases	percent
1st Return of menses	AFTER ONE MONTH	2	8 %
	AFTER 2 MONTHS	2	8 %
	AFTER 3 MONTHS	3	12 %
	AFTER 4 MONTHS	2	8 %
	AFTER 6 MONTHS	2	8 %
	NO RETURN OF MENSES	14	56 %

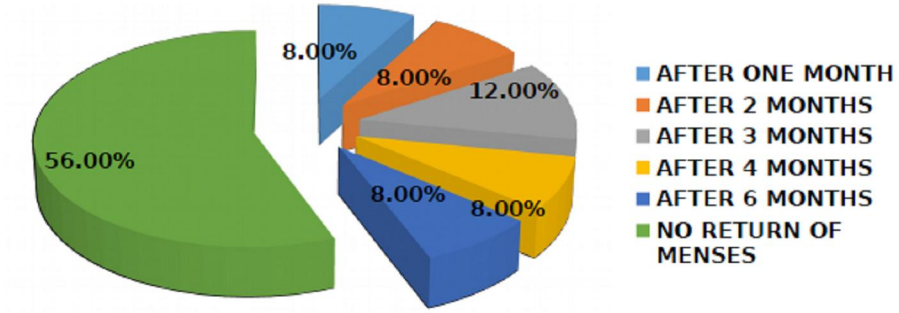

Figure 6: percentage of patients that resumed menstrual cycle 3rd outcome

	FSH	LH	E2	AMH	A.F.C
0 mon.	55	31	99	0.22	3
2 mon.	18.2	24	66	0.23	4
4 mon.	48.08	28	101	0.3	5
6 mon.	46.2	57	79	0.3	5
8 mon.	66	46	100.1	0.5	6
10 mon.	66	34	100	0.5	6
2 M after 2nd dose	53	47	46	0.5	6

⊡ Only one case got pregnant. She was 26 years old at the time of transplant, 1ry infertility, and 2ry amenorrhea 3 years. She had her 1st menses 3 months after transplant and just only one cycle. This table shows her follow up results over a period of 10 months. She received a second dose of stem cells laparoscopically 10 months after the 1st operation. Then 5 months after the 2nd dose, the patient was accidently discovered to be pregnant 9 weeks when she was investigated routinely for pregnancy test before doing chest X-ray for chest problem. She delivered a healthy female baby at 37 weeks gestational age by cesarean section.

⊡ We can't assert that stem cell transplantation has a role in this pregnancy as 10% of patients of POF may experience unexplained, unexpected ovulation.

4. Discussion

Many investigators have shown that bone marrow stem cells (BMSCs) could be promising grafts for the treatment of a variety of diseases, including reproductive dysfunction. [10], [11]. (Lee HJ, et al, 2007), (Ghadami M, et al, 2012).

In most patients with premature ovarian failure, especially during the earlier stages, the ovaries contain follicles, but the follicles are arrested before the antral stage. Development prior to this stage does not depend on gonadotropins because gonadotropin receptors are not present. Arrested development occurs at the stage when gonadotropin receptors are needed for further maturation.[12] (Nakano R, et al, 1978) So, Peri-menopausal women and patients suffering from primary ovarian insufficiency/failure have no antral follicles, but a limited number of primordial follicles are still present.[13] (Broekmans FJ, et al, 2009).

There are three major causes for premature ovarian failure, including X chromosome-linked

genetic defects, autoimmune disorders and long-term toxicity associated with chemotherapy exposure. Chemotherapy-induced premature ovarian failure is reversible. Mutations in the FSHR gene have been reported as a cause for primary ovarian failure (POF). Primary ovarian failure, also known as resistance ovarian syndrome (ROS), is a heterogeneous disorder characterized by amenorrhea and infertility in a normal karyotype female with an elevated serum level of follicle-stimulating hormone (FSH) and a decrease level of estrogen.

Hormonal replacement therapy (HRT) has been used to treat common menopausal problems, but it increases the risk of cancer or recurrence in cancer survivors; forcing physicians to use alternative treatments.

Fertility preservation is a rapidly evolving field that includes medical and surgical treatments to decrease the impact of cancer treatments on future fertility. Traditional fertility preserving techniques for patients undergoing radiation treatment included pelvic shielding or surgical repositioning of the ovaries out of the pelvis. Medical treatments to suppress ovarian function during chemotherapy have also been reported to decrease the effect on cancer treatments on future ovarian function. These modalities still rely on residual ovarian function after cancer treatments to conceive. Newer techniques to preserve ovarian reserve, oocytes, and embryos prior to cancer treatments have been developed to provide an opportunity to conceive in the event that cancer treatments result in permanent loss of ovarian function. **[14]** (Matthews M L, et al,2012)

Controversies on Neo-oogenesis

Greater numbers of studies have revealed that damaged ovarian function can be rescued after stem cell transplantation. Nevertheless, the mechanism behind this still remains unclear. Stem cells transplanted into the damaged ovary are more inclined to differentiate into oocytes or granulosa cell-like cells to replenish the lost granulosa cells, but could not develop into fully functional follicles in vivo. Additionally, factors produced by stem cells could inhibit stromal cell apoptosis, thereby playing a part in rescuing damaged ovarian function. [15] (Dan S, Haibo L, et al, 2014).

Mesenchymal stem cells (MSC) residing within the BM microenvironment are pluripotent adult stem cells, whose multipotency, easy isolation and culture as well as high ex vivo expansive potential make them attractive candidates for stem cell therapy. [16] (Imran Ullah, et al, 2015).

A re-colonization of adult human ovaries with new primary follicles requires their readiness by the means of the presence of nests of primitive granulosa cells. Such nests are required for follicular renewal, since superfluous oocytes are not preserved and degenerate. [17] (Bukovsky, et al, A 2004) In other words, even transplantation (transfusion) of autologous germ cells may not be sufficient for follicular renewal in aging women, which lack nests of primitive granulosa cells in their ovaries.[18] (Bukovsky A, et al, 2005).

Several studies have shown that bone marrow cells, or other normally circulating cells, are not involved in the formation of mature, ovulated oocytes. In 2005, Oktay et al, reported spontaneous pregnancy in presumably sterile human patient following chemotherapy, haematopoietic stem cell transplantation and ovarian tissue transplantation. At the same period Professor Bukovsky and his team at the University of Tennessee reported that they had obtained ovarian stem cells from five women aged 39 to 52 years. They cultured the cells for five days until they developed into eggs “suitable for fertilization. Eggan et al in 2006 used parabiotic mice to show that ovulated oocytes do not come from circulating stem cells, and also indicated that chemotherapy does not destroy all oocytes which may result in false result of follicular renewal. Begum et al, in 2008 tested the hypothesis that follicles can form de novo in adult ovaries in a transplant model. Ovaries from adult mice were transplanted under the kidney capsule or into the ovarian bursa of histocompatible, transgenic host animals. Some donors were sterilized before transplantation by X-irradiation to completely eliminate small oocytes and to ensure ‘empty niches’ were available for repopulation and screened at intervals by epifluorescence to ascertain whether they were repopulated with oocytes expressing GFP. None expressed green fluorescence, as would be predicted if they had formed de novo from germ cell progenitors in the systemic circulation of the host. Furthermore, small follicles eliminated by irradiation were not replaced in transplanted ovaries, and the few growing follicles present were apparently survivors of the original population. They conclude that No evidence was found to support the hypothesis that progenitor cells from extra-ovarian sources can repopulate the adult ovary. The findings are consistent with the conventional view that a limited number of oocytes are formed before birth and declines with age. The study did not, however, rule out the possibility that germline stem cells may reside in the adult ovary. [19] ***(Begum S, et al, 2008).***

Also, According to studies by *Liu Y, et al*, in 2007 and *Irma et al* in 2008 no evidence was found to support the hypothesis that progenitor cells from extra-ovarian sources can repopulate the adult ovary.

On the other hand, several studies proved that ovarian function and fertility could be restored after transplantation of different types of stem cells. Lee

HJ, et al, in 2007 concluded that bone marrow stem cells transplantation (BMT) rescued long-term fertility in CTX-treated female mice. This indicates the presence of unidentified factors/cells in BM, either stimulate resting follicles, not destroyed by the cytotoxic compounds, to enter growth phase, or initiate "putative" germinal stem cells, already present in the ovary, to undergo changes leading to a neooogenesis. However, maintaining the survival and self-renewal of transplanted seed cells in ovarian tissues over the long-term remains a troublesome issue. [20] (Lee HJ, et al, 2007). In 2008, Fu *et al*, demonstrated that BMT improves ovarian function in rats with chemotherapy-induced ovarian damage. [21] (Fu et al, 2008).

A study done by *Ghadami M,* et al, in 2012 to determine the effects of BMSC transplantation on reproductive physiology in female FORKO mice (follitropin receptor knockout), which is a suitable model to study ovarian failure in humans. Their results showed that intravenously injected BMSCs were able to reach the ovaries of FORKO mice, differentiate and express FHS receptor gene, make FSH receptor responsive to FSH, resume estrogen hormone production, and restore folliculogenesis. [11] **(**Ghadami M, et al, 2012).

Bhartiya D et al in 2015, reported the presence of very small embryonic-like stem cells (VSELs) in adult human testis and ovary that can survive chemotherapy because of their quiescent nature and can be detected in chemoablated mice gonads. Surviving VSELs spontaneously differentiate into oocyte-like structures and sperm when inhibitory factors are overcome **in vitro.** [22] ***(Bhartiya D, et al, 2015).***

Recent studies have shown the process of oocyte generation from ESCs (embryonic stem cells). [23] (Hayashi K. et al, 2013) and from induced pleuripotent stem cells (iPSCs). [24] (Goyal A, et al, 2013).

A study by Sun et al, in 2013 aimed to explore the therapeutic efficacy of ADSC (Adipose-derived stem cells) transplantation for chemotherapy-induced ovarian damage, the study revealed that ADSCs significantly improved ovarian function after chemotherapy-induced ovarian injury. [**25**] (Sun M, et al, 2013).

The objective of this study was to determine the effects of BM MSC transplantation on reproductive physiology in both female rats with chemically induced ovarian failure and human female patients with spontaneous premature ovarian failure. The treated animals showed improvement of the levels of the sex hormone E2, with homing of the iron tagged stem cells in the ovaries confirmed by histopathology. This current study supports the existence of stem cells in adult mammalian female's bone marrow that can influence ovarian function, even though BM transplantations in our experiment did not yield mature eggs following natural or induced ovulation. This may be due to the structural and functional interdependence within the follicle between the sex hormone-producing cells (granulosa, theca cells) and the oocyte.

Also, in this study 25 female patients of idiopathic POF were laparoscopically injected intraovarian with BM MSC. 20 patients were 2ry amenorrhea; some started to show initial improvement in the form of initial decline of serum levels of FSH and rise of E2, during the 1st 4 months of follow-up after transplantation. p- values for FSH, were non-significant, only one result was significant (P2: 0.023) that compare results of FSH after 2 months with that after 6 months of transplant. P values for E2 were non-significant throughout follow up. For AMH and AFC, p- values were significant when comparing levels after 2 months to that after 8 months (P3) and when comparing AMH levels after 4 & 6 months to that after 8 months of transplant.

Few patients showed minimal increase of AMH (4 patients) after 4 months, increased to 8 patients after 6months of transplant, but after that only 2 patients showed continued but very minimal increase of AMH, the maximum AMH level reached was 0.9 after 6 months of transplant raised from 0.2 before transplant. The P-value for both LH and AMH (< 0.001) is considered statistically highly significant. This actually means that BM MSC transplantation actually improved AMH which is produced by and easily detected in ovarian granulosa cells of early primary growing follicles, and hence improving the ovarian reserve.

Of total 25 patients, 11 had resumed their menses, representing (44%), all were cases of 2ry amenorrhea but unfortunately this was a transient return, with maximum No of cycles 7 irregular cycles, and a minimum of one cycle. This was associated initially with the relatively lowered FSH levels and increased E2 serum levels before starting to relapse again. Results during the first 4 months of follow up were very promising and cannot be ignored. Soon after, almost all cases that showed initial improvement started to relapse again when the FSH levels started to rise, E2 levels dropped back and menses stopped again.

Only one case got spontaneous pregnancy, she was 26 years old at the time of transplant, 1ry infertility, 2ry amenorrhea 3 years, and hypoplastic luteinized both ovaries by laparoscopy. She had her 1st menses 3 months after transplant and just only one cycle. She was retransplanted laparoscopically 10 months after the 1st operation. 5 months later, the

patient was accidently discovered to be pregnant 9 wks, when she was investigated routinely for pregnancy test before doing chest X-ray for chest problem. This was a spontaneous pregnancy, and she delivered a healthy female baby at 37 weeks gestational age by cesarean section.

This present study showed that BM MSC transplantation partially improved hormonal function of the ovary, steriodogenesis and to a lesser extent improves folliculogenesis in both spontaneous and induced POF in humans and rats respectively, as indicated by increased estradiol levels, follicle numbers, decreased FSH levels and return of the menstrual cycle in some patients. However this improvement was transient and followed again by an unexpected relapse of the serum hormones and cessation of the menstrual cycle.

Stem cells also may have a prophylactic action against ovarian damage by chemotherapy as shown in rats. One of the mechanisms behind this is the integration of MSC into the tissue and replacement of damaged cells; in addition, the paracrine mediators secreted by MSC might be involved in the repair by preventing cell apoptosis, inhibiting ovarian follicle atresia and promoting functional recovery, therefore increase their resistance to chemotherapeutics. The improved ovary after stem cell transplantation is a complex mix of many unclear factors requiring further investigation.

Conclusion

Results proved that intraperitonealy transplanted bone marrow stem cells homed in the damaged ovaries, improved the function of the chemotherapeutic damaged ovaries of female rats, and restored steroid hormone production. Stem cells also showed a prophylactic action against ovarian damage by chemotherapy.

Regarding the human part of the study, some female patients with spontaneous POF transplanted with autologous bone marrow stem cells showed initial improvement of the sex hormones E2, FSH and LH together with resumption of the menstrual cycle. However, this improvement was transient. One case of 2 that had repeated transplantation got spontaneous pregnancy after 4 months of repeating transplantation and delivered a healthy female baby, however it can't be asserted that it is due to the effect of stem cell therapy, as 10 % of patients with POF can have spontaneous unexpected ovulation. We believe that the return of ovarian activity was the result of incorporation of the stem cells within the ovarian tissue, promoting functional recovery through both autocrine and paracrine mechanism. However; maintaining the in vivo survival of transplanted cells in ovarian tissues over the long-term remains a challenge for greater clinical application in humans.

Recommendations

Understanding the complexity of recovery of ovarian function in POF after stem cell transplantation, cause of ovarian failure whether spontaneous or related to chemotherapy and how ovarian stem cells interact with their surrounding environment with determination of the MSC dose according to its biological effectiveness is a critical point for achieving a successful cell based therapy.

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