Bone-Marrow Derived Stem Cells Protocol for Premature Ovarian Failure – A case study with successful spontaneous pregnancy and delivery after treatment.

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Case Report

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Abstract

OBJECTIVES Stem cell-based therapies are emerging enormously as a ray of hope and therapeutic alternative for ovarian rejuvenation, regeneration and follicular development in women with impaired ovaries mainly due to primary ovarian insufficiency or premature ovarian failures, the conditions which cannot be improved much medically or surgically. In the modern society, the childbearing age is being delayed by women due to profound socio-economic changes making ovarian aging a key challenge for reproductive medicine. Based on the current scenario, the need of the hour is novel methods to restore fertility for all cases of ovarien impairement like poor ovarian reserve or primary ovarian insufficiency. As per the studies reported, the infusion of the whole population of human bone marrow-derived stem cells promote ovarian local vascularization, human follicle growth, increase follicle and stromal cell proliferation, and lead to reduction in apoptosis and atresia thereby, providing an adequate ovarian niche for follicular rescue in patients.

MATERIAL AND METHOD Based on the previous reports, we aimed to evaluate the effects of autologous bone marrow derived stem cell transplantation into ovary on the ovarian reserve of women with impaired ovarian functions. We, therefore, designed a method to deliver bone marrow derived stem cells directly to human ovaries.

RESULTS This study showed that autologous bone marrow derived stem cell ovarian transplant led to the improvement in ovarian reserve biomarkers and reproductive outcomes which results in more follicles and oocytes developed after ovarian stimulation. This technique allows successful pregnancies in women with impaired ovarian functions, however, further research is still needed as the embryo euploidy is not modified by the stem cells therapy. Diagnostic hormonal levels improved post procedure; there was a reduction in FSH and increase in AMH and estradiol levels.

CONCLUSIONS Stem cells are foundation cells of the body with the remarkable abilities of self-renewal and differentiation into other cell types and this treatment bring hope to a lot of couples dealing with infertility.

Introduction

Several factors such as lifestyle changes, social causes, work, and economic scenarios have led to the trend of delayed motherhood despite the biological clock that keeps ticking. Females in their 20s have the best reproductive potential but not many women choose to be mothers during this age. The quality of oocytes produced in 20s is the best which increases the chances of a safe pregnancy that will result in a healthy baby. However, it has been estimated that premature ovarian failure (POF) or early menopause affects 10% of the female population, who despite the young age, will have problems to conceive. [1, 14]

Primary ovarian insufficiency (POI) of premature ovarian failure (POF) is a condition marked b the loss of normal function of ovaries before the age of 40. Ovarian failure implies that the eggs are not regularly released which results in infertility (2,3). Many women naturally experience reduced fertility by 40 years of

age. This age may mark the start of irregular menstrual cycle that signals the onset of menopause. Women with POI experience early menopause which is denoted by irregular periods and reduced fertility before 40 years old, sometimes as early as the teenage years (4,5)

The first sign of POI is usually menstrual irregularities (6), also called amenorrhea. The other symptoms may include occurrence of hot flashes, night sweats, poor concentration, irritability, decreased sex drive, vaginal dryness, and pain during sex which are consistent with the symptoms of naturally occurring menopause (7,8). For most of the women with POI, trouble getting pregnant is the first symptom they experience which leads them to visit their health care provider. This is sometimes called “occult” (hidden) or early POI (9), which is usually characterized by amenorrhea, low Anti-Mullerian Hormone (AMH) and estradiol levels, oligomenorrhea, hypoestrogenism, and elevated gonadotrophin and follicular stimulating hormone (FSH) levels in women under the age of 40 (10,11,14).

The cause of POI can be natural or it can result after surgery, disease, chemotherapy, and/or radiation. With POI, some women may still have occasional periods and they may even get pregnant. Evaluation of ovarian reserve and personalisation of the therapeutic approach are crucial for optimizing the success rate for its treatment.

Various study groups have tried to restore fertility in women with POI; however, very few randomized therapeutic trials registered show significant improvement in ovulation and pregnancy rates. In a systematic review about various therapeutic interventions with a purpose to restore ovarian function in women with POI, the authors concluded that most of the interventions were equally ineffective and unlikely to project an improvement in the prevailing scenario.

Nevertheless, it was shown that stem cells derived from varied sources may rescue the ovarian functions, such as recovery of ovarian sex hormone function, reduction in apoptosis of germ cells, and increment in the number of follicles.[12] However, the improved ovary function after stem cell transplantation is a complex mix of many unclear factors requiring further investigation. [13, 14].

According to earlier reports, spontaneous pregnancies were successfully achieved after bone marrow transplantation in oncologic women with POI. Stem cells derived from varied origins promote follicular development, increase follicle and stromal cell proliferation, increase ovarian local vascularization, and reduce cell apoptosis and follicular atresia, while keeping the embryo quality intact (14). Hence, if the ovarian environment is adequate, residual quiescent follicles of damaged or aged ovaries can potentially produce competent oocytes. Nevertheless, further research is required to properly evaluate underlying mechanisms, identify best cell sources and design less/ minimally invasive infusion techniques. This study shows that stem cell therapy can potentially be used as a relevant therapeutic alternative for ovary regeneration and follicular development in patients with impaired ovaries (14).

Overview Of POF:

Definition

A normal healthy woman menstruates regularly from menarche – her first period- (usually occurring at 10–14 years old) until menopause (usually that was taking place at 50–55 years old). Nowadays, women have their menarche much sooner (7–9 years old) and the ovaries stop functioning before the age of 40, pathology known as premature ovarian failure (POF)– also called Primary ovarian insufficiency (POI). (15)

Prevalence

POI affects approximately one in 1,000 women under the age of 30 and one in 100 women under the age of 40.and is characterized by menstrual disturbance (amenorrhea or oligomenorrhea) with raised gonadotropins, low anti-Mullerian hormone (AMH), the ovaries do not produce normal amounts of the hormone estrogen and do not release eggs regularly. This condition leads to infertility.(16)

Signs and Symptoms

Women with POF were found to have a wide variety of signs and symptoms affecting many body systems due to hypoestrogenism and hypoandrogenemia, that may lead to serious diseases. Main symptoms include hot flushes – that especially appear prior to the onset of amenorrhea, vasomotor symptoms associated with sleep problems which may affect social and work performance, urogenital symptoms as vaginal dryness, vaginal irritation, and itching, cardiovascular disease due to abnormal lipid profile, insulin action disturbances as patients with POI have a higher risk of developing type 2 diabetes mellitus as compared to women with normal menopause. In addition to these, peak bone mass (PBM) formation and bone mineral density (BMD) status were found to be affected at younger age and with more severity. (17)

Pathogenesis

According to pathogenesis, there are two types of POF: one has an abundant number of follicles but with maturation defects and the other type has limited number of remaining follicles. The POF pathophysiology is believed to differ from normal menopause. The declined ovarian function in the first type of POF is reversible, whereas in the latter one the changes are permanent.

However, the pathogenesis of premature ovarian failure is not entirely clear and it requires further investigation, but until now, there have been described three major causes for POF.

The first cause for POF is based on X chromosome-linked genetic defects, which include X monosomy (also called Turner's syndrome), trisomy X7, mosaicism and X chromosome deletions. Most of the genetic studies in POI were conducted on genes already known to play a role in folliculogenesis (*NR5A1*, *NOBOX*, *FIGLA*, and *FOXL2*), as folliculogenesis growth factors (*inhibin A*, *GDF9*, and *BMP15*), or in ovarian steroid genesis (*FSHR*, *FSH*, *LHR*, and *LH*). (17)

Karyotype testing should be always recommended, as it was shown that approximately 5–10% of women with a diagnosis of POI that have a normal karyotype, can achieve a spontaneous pregnancy even if the

recovery of ovarian function is temporary and poorly predictable (18)

The second cause for POF is represented by autoimmune disorders, associated with humoral and cellular immunity modifications, resulting in antibody creation and T-cell mediated injury of ovarian granulosa cells (GCs), oocytes and the zona pellucida. Based on the data from the St. Marianna University School of Medicine and the Rose Ladies Clinic, 45% of the patients with idiopathic, normal karyotypic POI, tested for 16 autoantibody tests (Mentioned in paper). were found to have positive results. From these, forty-one patients (15% of patients with positive autoantibodies) were diagnosed with clinical autoimmune diseases. The autoimmune disorder most frequently associated with POI, as mentioned in the paper, was hypothyroidism, followed by hyperparathyroidism.

The third most important cause for POF is iatrogenic, due to ovarian surgery, chemotherapy and radiotherapy (cancer survivors). (17)

In addition to this, it is worth mentioning that the Chemotherapy-induced premature ovarian failure may be reversible and the patient may gain back her fertility. One report suggests that the younger females who suffered from chemotherapy- induced POF still retain enough ovarian function with good quality oocytes to support a successful pregnancy (18).

In addition to these 3 major causes, it has been shown in case reports that viral infections can be followed by ovarian failure. However, only mumps oophoritis has been considered to be a cause of POI, accounting for 3–7% of POI cases (17)

Overview Of Stem cells:

Stem cells are a population of undifferentiated cells characterized by the pliability to extensively proliferate and differentiate into differing types of cells and tissues. Stem cells have many sorts, that differ from each other by their origins and derivatives.(19) Pluripotent cells which are derived from the inner cell mass of the embryo and induced pluripotent cells can differentiate into tissue from all three germ layers.(20) Multipotent stem cells may become multiple specialized cells in an exceedingly specific tissue like hematopoietic stem cells (HSC) and mesenchymal stem cells which form tissue, bone, and cartilage. Unipotent or oligopotent stem cells will only differentiate into one cell type, since they will form terminally differentiated cells of a selected tissue. (21)

There are many applications of stem cells nowadays, starting from cellular therapy that aims to exchange damaged cells and regenerate organs. (22) Also, stem cells have expanded our understanding of development and the pathogenesis of diseases. (23) Nowadays, there is a big development within the field of somatic cell and regenerative medicine that have driven basic, translational, and clinical advances.

Mesenchymal stem cells (MSCs) are considered to be a relevant therapeutic alternative for ovary regeneration and follicular development in patients with impaired ovaries, like women diagnosed with primary ovarian insufficiency.(24) Although adult stem cell treatments do not modify the embryo quality,

they have achieved a golden role in this field, by promoting follicular development, increasing ovarian local vascularization, enhancing follicle and stromal cell proliferation and reducing cell apoptosis and follicular atresia.(25) Following transplantation of stem cells, clinical trials have shown that menstrual cycle and even pregnancy may occur in POF patients.(26, 27) Nevertheless, we should have in mind also the restrictions, including inadequate number of cases reported, and the need for the development of transplantation methodology. In addition to this, we should remind that immunogenicity, heterogeneity and stem cells inherent properties of tumorigenicity are all considered challenges that may encounter in this promising field.

Case Presentation

We present the case of a 32-years-old woman, diagnosed with idiopathic POF at the age of 28 years old, married and trying to conceive for one year.

The main clinical manifestations were irregular menstruation, hot flushes, vaginal dryness, accompanied by hormonal imbalance for 2 years (FSH = 44 mIU/mL LH = 15 mIU/mL AMH = 0.01) but with a normal TSH. A transvaginal ultrasound showed a normal sized uterus with bilateral atrophic ovaries, so no follicles were counted. She hasn't done karyotype test because she had normal menarche and regular cycle and her condition is considered a secondary amenorrhea with free family history. Previously, she has gone through 2 IVF cycles but failed, and she was on multi-vitamins.

After complete evaluation, we performed a laparoscopic ovarian injection of BM stem cells and endometrial BM stem cells therapy based on O-Cell® Protocol of StemGenn Therapeutics by Prabhu Mishra on 5th of MARCH 2020 after she had oligomenorrhea for 4 years and amenorrhea for the last 2 years. We injected 2cc of BMSC in each ovary and 1cc in the endometrial cavity by endometrial cavity. By laparoscope uterus was normal, both ovaries are atrophic, no adhesions and adnexa were normal.

After the procedure, the patient was given Cialis (Tadalafil which increase blood flow to uterus and ovaries) 5 mg half tab daily for 30 plus dexamethasone 0.5 mg tab daily for 30 days and myoinositol 2000 mg once daily for 3 months.

One month after the injection, the patient received 3 sessions of intravaginal radiofrequency treatment, one session per month for three consecutive months.

3 months after intraovarian injection of BM stem cells the patient received transvaginal platelet rich plasma (PRP) 2cc in each ovary along using ovum pickup catheter under ultrasound guidance. We used Regan lab kit 10 cc of blood in the tube, centrifuged for 5 minutes on 3400 rpm rate.

4 months after intraovarian injection of BM stem cells, the ultrasound only revealed a slight enlargement of each ovary to measure 2 cm in diameter with 1 good antral follicle (10 mm) with many small follicles. The patient had no menstrual cycles and she refused repeating the hormonal profile for financial reasons. Nevertheless, her symptoms of hot flushes and vaginal dryness improved.

6 months after intraovarian injection of BM stem cells the patient received another injection of intraovarian BM stem cells, as the same procedure of the first trial under transvaginal ultrasound guidance. And she continued taking Myoinositol 2000mg once daily

7 months after the first injection, the patient had her first menstrual cycle which was a brownish minimal discharge of one day duration (late September). Her FSH level decreased to normal levels (9 mIU/ml) but the patient could not afford AMH measurements.

8 months after the first injection the menstrual cycle became regular with menstruation lasting five days. (Late October). 9 months after the BM first injection the menstrual, she had another physiological menstrual cycle. (In November). The patient, along with her health care provider, decided to try conceiving in December.

The patient was followed up by an ultrasound scanning for follicular measurements with no ovum stimulation.

Once one of the follicles happily reached 20mm, the patient was advised to try sexual intercourse. Also, another follicle was measured 14 mm and more than 4–5 small follicles were found in each ovary.

After 9 months from the BM injection, the patient presented for 6-week amenorrhea and the ultrasound showed intrauterine gestational sac with positive fetal heartbeat. The pregnancy presented no fetal complications, but the patient developed gestational diabetes in the last trimester, that was controlled with diet and metformin 850 mg per day.

The delivery was a by c-section at 38 weeks for obstetrics indications (CPD) due to cephalopelvic disproportion.,

We were happy to have a healthy baby boy of 3.5 kg with normal development in the extrauterine life.

Procedure Followed –

In this prospective study of 10 women defined as PRs using the ESHRE criteria, autologous bone marrow derived stem cells were delivered directly to both the ovaries for each patient in an effort to optimize the recruitment of existing dormant follicles to improve IVF outcomes. Patients with POF, POI or Low Ovarian Reserve willing to try the possibility of improving their ovarian function with informed consent underwent rejuvenation of premature ovarian failure with autologous bone marrow derived stem cells

They underwent diagnosis and screening confirming including history and physical exams, laboratory and diagnostic procedures shown in Fig. (3).

Following final approval, 45–60 mL of bone marrow aspiration was done from the patient's iliac crest, under general anesthesia maintaining strict asepsis using a Jamshidi needle prewashed with heparinized medium. Collection of the aspirate was done in CPDA medium using 1ml of the medium for 7ml of the bone marrow in 15ml centrifuge tubes. The sample was processed manually using a centrifuge by the

standard double spin procedure. Sample was centrifuged immediately at 1800 rpm for 12 min to separate the red blood cells. The plasma and buffy coat were transferred again to fresh tubes and centrifuged again at 2200 rpm for 5 min to obtain the bone marrow derived mono-nuclear cells. Then, 2-2.5 ml of cells were injected into each of the ovaries through laparoscopy.

Results & Discussion

Shortly after, patients then proceeded with controlled ovarian hyperstimulation for IVF with preimplantation genetic screening. Main endpoints included clinical improvement of ovarian reserve as measured by antral follicle count (AFC) and anti-müllerian hormone (AMH) levels, monitored up to five months after autologous bone marrow derived stem cell therapy, as well as cycle number (cancelled and completed), number of mature oocytes retrieved, number of euploid embryos, and pregnancy and live birth rate (including spontaneous pregnancies). An additional component of the study looked at growth factors released by autologous bone marrow derived stem cell therapy.

Results after autologous bone marrow derived stem cell therapy were promising for PRs. Autologous bone marrow derived stem cell therapy resulted in a significant improvement in AFC after treatment. The team defined success as an increase in AFC $\geq$ 3 follicles and/or two consecutive increases (two standard deviations) in AMH levels, and with this criteria ovarian function improved in of women. These positive effects were associated with the presence of fibroblast growth factor-2 (FGF-2) and thrombospondin (THSP-1) in the aphaeresis sample. Among the 10 patients, six pregnancies were achieved: 5 after embryo transfer and 1 by natural conception. This corresponds to a 60% pregnancy rate in a group of poor prognosis women where oocyte donation was the only practical option after several IVF attempts and years of infertility. It should be noted that after preimplantation genetic screening, the embryo euploidy rate was low.

Conclusion

· Improvement in diagnostic hormonal levels (Reduction in FSH and increase in AMH and estradiol levels).

· Resumption of menses.

· Improvement in hormonal levels toward normal ranges. Hormones may include FSH/LH; Estradiol/progesterone; Inhibin; Anti-Mullerian Hormone.

· Achievement of pregnancy by natural or assisted conception methods as may be deemed appropriate by the patient and her primary provider.

Declarations

Author Contributions: Conceptualization, P.M., I.N. and S.A. resources, P.M. and S.A., L.A. and S.A. writing—original draft preparation, P.M., I.N., S.A., M.A. and S.A. writing—review and editing L.A., D.M., P.M. and S.A. visualization, P.M., I.N and S.A. supervision, P.M., D.M. and I.N. administration, P.M. and I.N.

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Data Availability Statement: All data generated and analyzed in the study are included in the published article.

Conflicts of Interest: The authors declare no conflict of interest.

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Figures

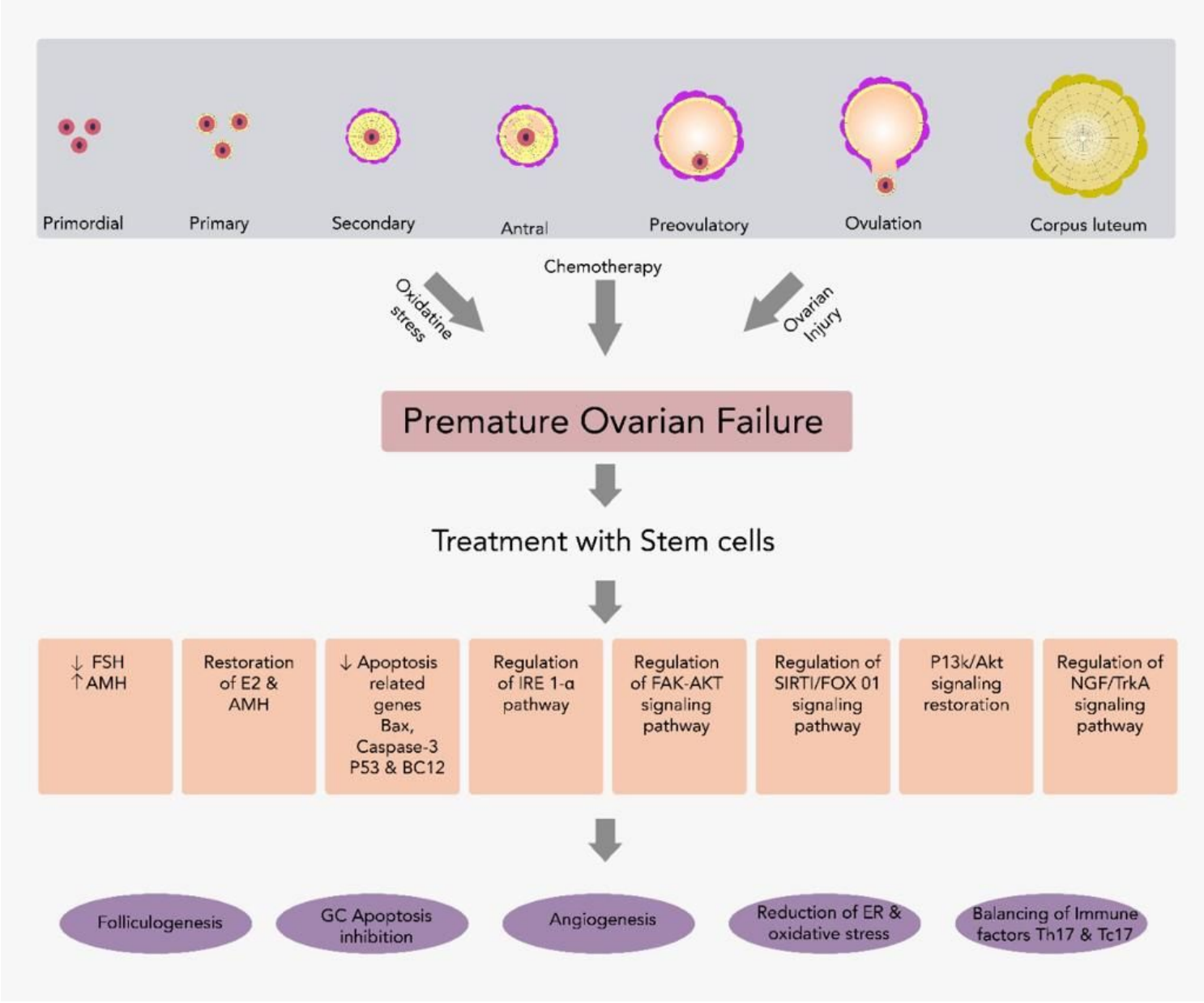

Figure 1

How stem cells work in premature ovarian failure and mechanism of action.

Figure 2

2 a Bone Marrow Aspiration.

2 b Bone Marrow Harvesting Requirements.

2 c Bone Marrow Aspired Concentrate Harvested by Double Centrifugation Method.

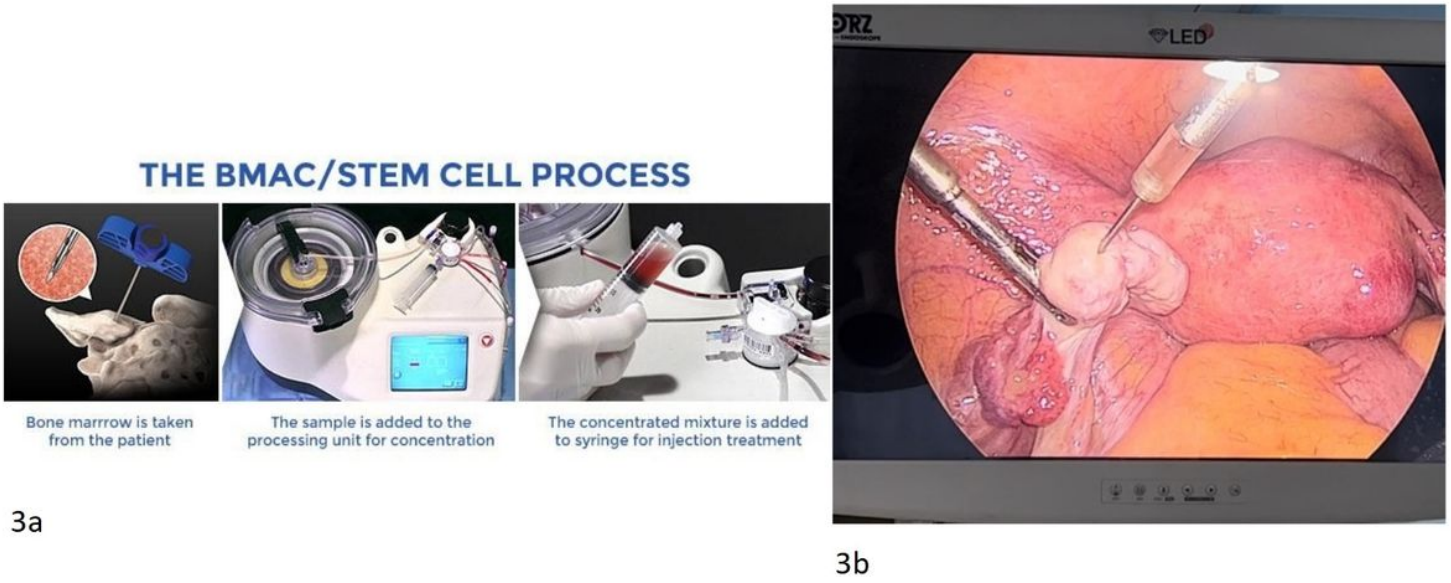

Figure 3

3 a Double Spin Centrifugation procedure.

3 b Laparoscopic Injection of Cells in Ovaries