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Endometrial regeneration in Asherman's syndrome and endometrial atrophy using Wharton's jelly-derived mesenchymal stem cells

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ABSTRACT

Objectives: Reconstruction of the endometrium in patients with endometrial atrophy and Asherman's syndrome using Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs).

Material and methods: Prospective pilot study, with the inclusion of two patients.

Results: After administration of WJ-MSCs into the uterine cavity, endometrial reconstruction was achieved in both patients. Pregnancy was achieved in one of them, after transfer of a frozen embryo, completed by delivery around the due date.

Conclusions: Endometrial atrophy and Asherman's syndrome, is one of the most frustrating clinical situations we face in assisted reproductive procedures. The use of Wharton's jelly-derived mesenchymal stem cells in restoring the normal function of the endometrium, could become an easy and accessible therapeutic medal, for this endometrial dysfunction, which is so difficult to treat.

Key words: endometrial regeneration; endometrial atrophy; Asherman's syndrome; stem cells; mesenchymal cells; procreation; Wharton's jelly

INTRODUCTION

The ability to fertilize, develop a pregnancy and give birth to a 20-year-old healthy woman is relatively low and at 20% per cycle [1]. For a woman to become pregnant, not only the gametes (egg cell and sperm) are needed but also the appropriate environment in which the fertilized egg can grow. This environment is the endometrium [2].

To date, the exact mechanism by which the embryo is implanted in the endometrium is not known. The most popular theory is that this happens through signals sent from the embryo to the endometrium and *vice versa*, while suppressing the immune system [3]. A necessary element of this process is proper reactivity of the uterine mucous membrane, which we monitor by measuring its thickness in a transvaginal USG examination [4]. With the use of molecular diagnostics and the expression of 238 genes present on the surface of the endometrium, an individual assessment of the "implantation window" has become possible. Performing embryo transfer at the most optimal time for the embryo has become the basis for increasing the effectiveness of the in vitro fertilization procedure [5].

Lack of growth of the endometrium and, consequently, the lack of its reactivity results in repeated embryo implantation failure, a situation in which a properly formed and healthy embryo does not implant in the uterine cavity [6].

The existing methods of therapy involve:

- hormone replacement therapy (estrogen administration);
- improvement of vascular flow by phosphodiesterase type 5 inhibitors administration (Sildenafil)- endometrial scratching;
- controlled damage to the endometrium to stimulate its reactivity;
- surrogacy — transfer of the embryo into the body of another woman (a method prohibited in Poland).

The methods described above did not give expected therapeutic effects and with variable luck they are propagated as the leading ways to stimulate endometrial growth [6, 7]. In case of iatrogenic damage to the endometrium, the standard treatment for years is

hysteroscopic repair [8–10, 11–13]. Malformations within the uterus are one of the causes limiting fertility. It is estimated that they constitute about 5–10% of causes of female infertility [14–16]. In this group, between 2% to 20% of women suffer from Asherman's syndrome or intrauterine adhesions [7, 8]. The term "mesenchymal stem cells" (MSC — mesenchymal stromal cells) is defined as multipotent progenitor cells with the ability to differentiate and mature into cartilage, bone and fat cells [17]. They perform an auxiliary function for other stem cells in the production of connective tissue of particular organs and form the stroma of bone marrow for hematopoietic cells. They also have the ability to modulate functions of the immune system [18]. According to the classification introduced by the International Society for Cellular Therapy, MSC should fulfill three conditions: have the ability to grow in vitro in the adherent form, have certain surface antigen expression such as CD13, CD44, CD90, CD73, CD105 with lack of CD14, CD11b, CD79, CD34, CD45 and HLA-DR and be able to differentiate into bone, cartilage and fat tissue [19, 20].

Cells meeting criteria for MSC can be isolated from a number of tissues of both germinal and fetal origins as well as from adult donors [21–25]. In recent years, the most frequently used source of MSC obtained from adults has been bone marrow (BM-MSC — bone marrow derived mesenchymal stromal cells). However, the obtained cell populations were very heterogeneous, of which hematopoietic stem cells were the majority with much smaller percentage of BM-MSC. Recently, adipose-derived stem cells (ADSC) have been gaining popularity. These cells are much more homogeneous cell group than BM-MSC. In addition, the occurrence of MSC in the bone marrow has been demonstrated to be between 1:2,500 and 1:100,000, and ADSC in the adipose tissue population 1:50.

At the fetal stage of development, MSC are present in the blood of the fetus (umbilical cord blood), Wharton jelly, perivascular region, submucosa, umbilical cord, placenta and amniotic fluid. In contrasts, clinically used MSC taken from umbilical cord and Wharton jelly are characterized as easily collectable appropriate amounts of material without ethical issues.

WJ-MSC jelly meet the criteria described above: they are self-renewing and can differentiate into different tissues, not only bone, cartilage and fat, but also into striated muscle cells [26], cardiomyocytes [27], hepatocytes [28, 29], pancreatic Langerhans cells [30–32] and nerve cells [33, 34]. They can also take part in the regeneration of retinal structures [35]. They have human leukocyte antigens class I (HLA-I) on their surface but they do not have HLA class II surface antigens [36–38]. They induce immunosuppressive effect on

lymphocytes and inhibit T cell proliferation [39]. The above-mentioned immunological properties of these cells result in both lack of reactions of the recipient's immune system to mesenchymal cell transplantation as well as lack of reaction of allogeneic mesenchymal cells to function in the recipient's tissue system. It is believed that stem cells act as a local tissue repair coordinator. The repair of damaged tissue occurs by regulating endogenous regenerative processes and not by replacing damaged tissue with de novo structures originated from mesenchymal cells [40]. Fetal MSC have greater expansion potential in *in vitro* culture than adult mesenchymal stem cells.

Many authors believe that this is a consequence of two passages of primary hematopoietic cells from the embryo to the placenta and back [41]. The first passage, between 4 and 12 days of embryogenesis, starts through a primitive umbilical cord and hematopoietic cells migrate from the yolk sac to the placenta. Then during the second migration, the MSC are passed in the opposite direction. They return from the placenta to the fetus: to the liver and then to the bone marrow [42]. Researchers believe that during this migration, the mesenchymal cells are trapped in Wharton jelly, where they stay from early embryogenesis throughout pregnancy until delivery. WJ-MSC can interact in the organism of the donor in three independent mechanisms: differentiation into different types of tissues, through the immunomodulatory effect and through the ability to regulate external effects occurring in the environment of MSC, through the secretion of appropriate cytokines and direct contact with other cells. According to recent scientific reports, the most important role of MSC is considered to be the local coordination of tissue repair. The result of interaction between MSC, other cells and tissue regeneration mediators, can possibly adapt MSC signaling to the changing situations. It has been shown, however, that the structures directly created by posterior cells derived from MSC are relatively rarely responsible for the repair of the damaged area [17].

MATERIAL AND METHODS

Our research project is based on the already obtained scientific knowledge, described in more than 300 publications on the use of MSC in human repair processes. Every organ of the human organism to a greater or lesser extent has the ability to regenerate. A logical observation is that the endometrial ability has such ability, since it exfoliates during menstruation with a regularity of about 28 days. A breakthrough in the field of regenerative medicine within the mucous membrane of the uterine cavity was a scientific report by Taylor [42] — describing the regeneration of endometrial cells in patients after bone marrow

transplantation, in which during preparation for donor bone marrow cell administration, the mucous cells of uterine cavity are being damaged iatrogenically and loss of its functionality. Based on this discovery, the possibilities of endometrium regeneration began to be studied. The first scientific report describing the use of autologous stem cells in a patient with Asherman's syndrome was published in 2011 [43]. After administration of MSC to the uterine cavity, the endometrium increased above 7 mm, which allowed embryo transfer and development of an intrauterine, single pregnancy. Recently Santamaria et al. [44] described the procedure for the administration of autologous stem cells derived from the bone marrow to the uterine spiral arteries in 16 patients with Asherman's syndrome and endometrial atrophy [44]. All patients underwent endometrial regeneration and four of them became spontaneously pregnant. Stem cells derived not only from peripheral blood or from bone marrow [44, 45] but also from menstrual blood can be used for regeneration of the endometrium [46].

Umbilical cord collection, cell isolation and culturing All Umbilical Cord (UC) samples were obtained after patients provided informed consent, ethical approval was given by Bioethical Committee. UC were collected after natural delivery as well as caesarian sections. Transport conditions were monitored and tissue was processed within 48 h of delivery. Umbilical cord fragments were washed in a sterile saline with Antibiotic-Antimycotic solution (Gibco). Then UC was dissected and blood vessels were removed. Wharton Jelly was minced into 2 mm scraps and placed into culture flasks covered with MSC Attachment Solution (Biological Industries) according to manufacturer's recommendations and grown in serum free medium for human mesenchymal stem cells NutriStem® XF (Biological Industries) with NutriStem ®XF Supplement Mix (Biological Industries) with the addition of Antibiotic-Antimycotic solution (Gibco). Culture was incubated at 37 C in 5% CO2 in the air. Tissue explants were removed after 2–3 weeks of the culture. Adherent cells were passaged upon reaching 90% confluence and reseeded at 1.2 × 104 cells/cm^2 for further expansion. After trypsinization with Tryple solution (Biological Industries) number of cells were evaluated. When the required number of cells was obtained, they were transferred into a freezing bag and resuspended in 5% solution of human serum albumin (CSL Behring) in the presence of 10% DMSO (WAK-Chemie link), cooled down with controlled rate freezer and then placed in the vapor phase of liquid nitrogen Viability assay Viability was determined based on the thawed reference sample and counted by the trypan blue exclusion in hemocytometer. Immunophenotyping of human umbilical cord MSC Characterization of

human umbilical cord derived mesenchymal stem cells (hUC-MSC) was carried out with accordance of minimal criteria of mesenchymal stem cells described elsewhere (Dominici et al. [19]) by immunophenotyping using both MSC-positive and MSC-negative surface markers. Briefly, 60 to 80% confluent flasks of expanded MSC were trypsinized and then incubated with following antibodies in the dark for 30 minutes. Cells were stained with antibodies against: CD34 FITC, CD14 FITC, CD19 FITC, CD 45 FITC, HLA-DR FITC as a negative marker, CD 73 PE, CD90 PE, and CD105 PE. Then cells were acquired and analyzed using a BD FACS CALIBUR cytometer equipped with 488 nm argon-ion laser.

Study design

This first pilot research project was approved by the Bioethics Committee at Centre of Postgraduate Medical Education in Warsaw (67/PB/2016) and sponsored by the Polish Stem Cell Bank in Warsaw. Two patients treated for infertility were included in the study. The first with Asherman's syndrome due to previous curettage of the uterine cavity as a result of postnatal hemorrhage. The first patient was initially twice subjected to hysteroscopic treatment of cutting intrauterine adhesions and then treated with HRT. The patient three times underwent frozen embryo transfer, without getting pregnant. The second patient was diagnosed with endometrial atrophy due to a double resectoscopic dissection of the septum in the uterine cavity.

Both patients were qualified for the study and operated by the same medical team. During menstrual bleeding, both underwent uterine cavity curettage and followed by ultrasound –guided injection of WJ-MSC in a 1 ml saline suspension into the uterine cavity. In order to accelerate the regeneration and growth of the endometrium, both patients received hormonal supplementations within one month and underwent the procedure without any complications.

RESULTS

In the patient with Asherman’s Syndrome, before the MSC procedure, the endometrium was uneven and its width was between 3 to 5 mm (Fig. 1). During the first uterine curettage, a material was obtained that was examined and described by the histopathologist: fragments of the endometrium with features of prolonged proliferation in the form of small polyps. Fragments of the basal endometrial layer were hormonally non-reactive. One month after WJ-MSC administration, an increase in endometrium up to 7.6 mm was observed in ultrasound examination (Fig. 2).

The procedure of obtaining material from the uterine cavity was performed again. Histopathological examination revealed the endometrium in the initial phase of secretion. According to the patient's report, there was no significant difference in the duration of menstrual bleeding or its abundance in relation to the situation before the MSC administration. The patient re-joined the assisted reproductive treatment. As a result of the frozen embryo transfer, she became pregnant and delivered the baby with a Caesarean section at 34 weeks of pregnancy due to premature labor. The second patient had endometrium 3 mm wide before the MSC procedure in the control USG. In the histopathological examination of curetted material from the uterine cavity, a conclusion has been formed: endometrial fragments from basal layers were non-reactive hormonally. After one month of the MSC intrauterine administration the ultrasound examination showed an increase of endometrium width to 7.6–8 mm (Fig. 3).

In the material obtained from the re-curettage of the uterine cavity, the histopathologist identified fragments of the endometrium with features of prolonged proliferation. The patient reported that after the MSC administration, menstrual bleeding increased, and its abundance increased. The patient underwent assisted reproductive treatment and did not become pregnant due to the accompanying male factor.

DISCUSSION

Endometrial atrophy and Asherman’s syndrome are diseases that prevent procreation for women who have been subjected to pre-operative obstetric or gynecological procedures. The patients we qualified for this first pilot study of the allogeneic Wharton’s jelly-derived mesenchymal cells regenerative potential in the regeneration of the endometrium, had previously suffered iatrogenic endometrial trauma.

After MSC administration, endometrial hyperplasia was observed already in the first cycle, which was documented not only in the collected medical history of the menstruation but also in the change of endometrial thickness in transvaginal ultrasound examination, as well as histopathological examination of endometrial scrapings obtained before and after MSC administration. Particularly significant is the therapeutic effect obtained in the first patient who had previously performed three unsuccessful transfers of frozen embryos. After the endometrium regeneration and after the next frozen embryo transfer, she became pregnant and gave birth by the Caesarean section.

In the second patient, the intrauterine MSC administration caused permanent repair of the mucous membrane of uterine cavity, observed in the period of one and a half years from the medical procedure (the patient remains under constant care of our team).

Despite the fact that she did not become pregnant (the accompanying male factor of infertility), after each menstruation, the correct endometrial image is observed in the ultrasound examination, without the use of any hormonal therapy. The use of WJ-MSC seems to be a promising way to regenerate the mucosa of the uterine cavity. The use of allogeneic material in both patients did not cause any side effects. This safety was also confirmed by histopathological examination of the obtained uterine scrapings after MSC

administration. Therefore, it seems that the procedure of administrating allogeneic mesenchymal cells, in addition to allogeneic cells from bone marrow, peripheral blood or adipose tissue (not yet published), is a promising method of obtaining endometrial regeneration and may be an indispensable element of infertility treatment in the described groups of patients.

CONCLUSIONS

Endometrial atrophy and Asherman syndrome, is one of the most frustrating clinical situations we face in assisted reproductive procedures. The use of mesenchymal stem cells in restoring the normal function of the endometrium, could become an easy and accessible therapeutic medal, for this endometrial dysfunction, which is so difficult to treat.

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Conflict of interest

All authors declare no conflict of interest.

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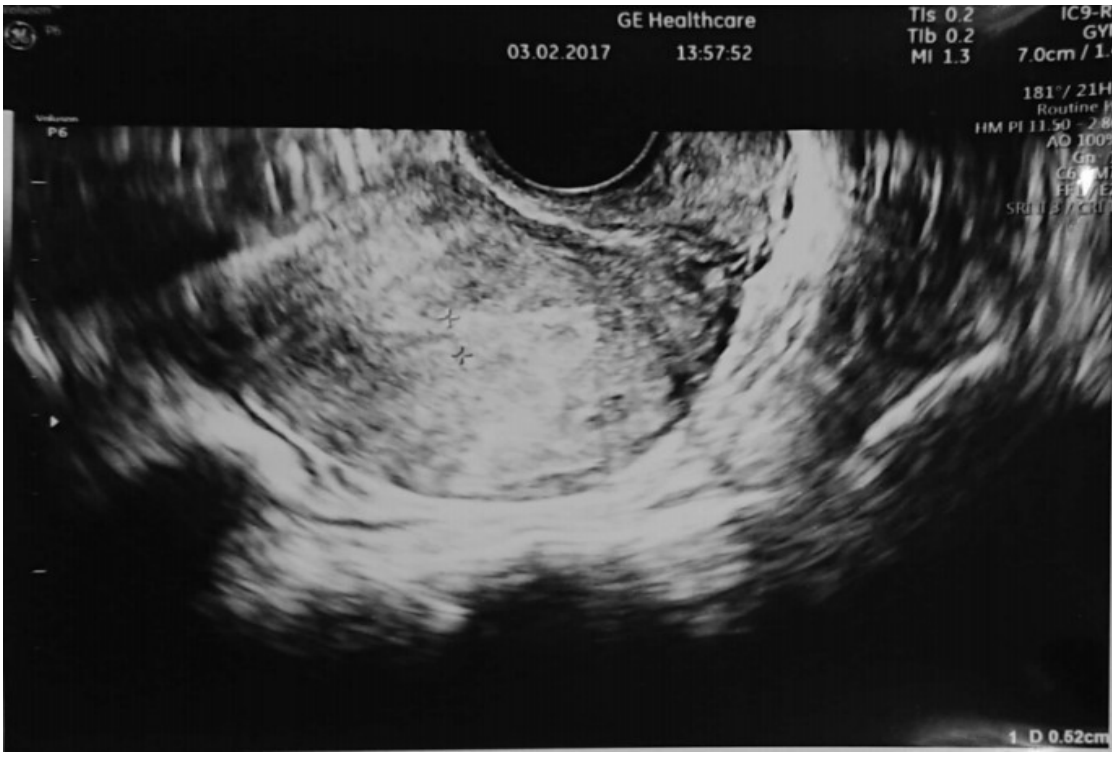


Figure 1. Endometrium of the first patient before the procedure

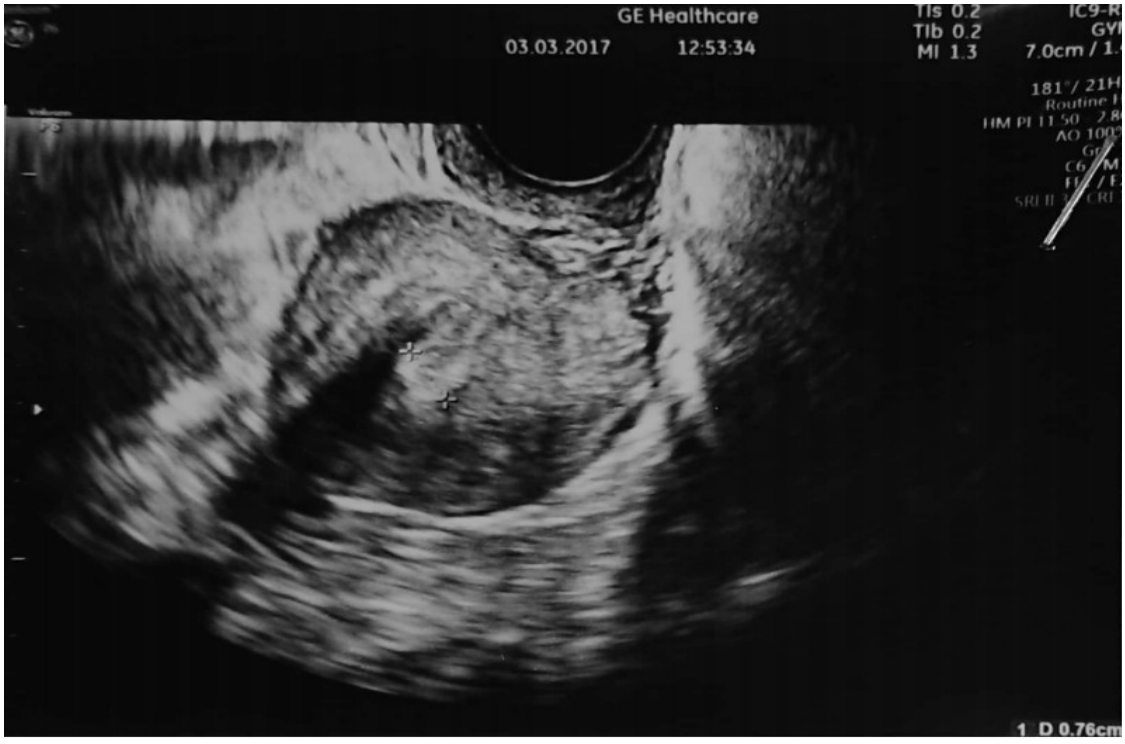
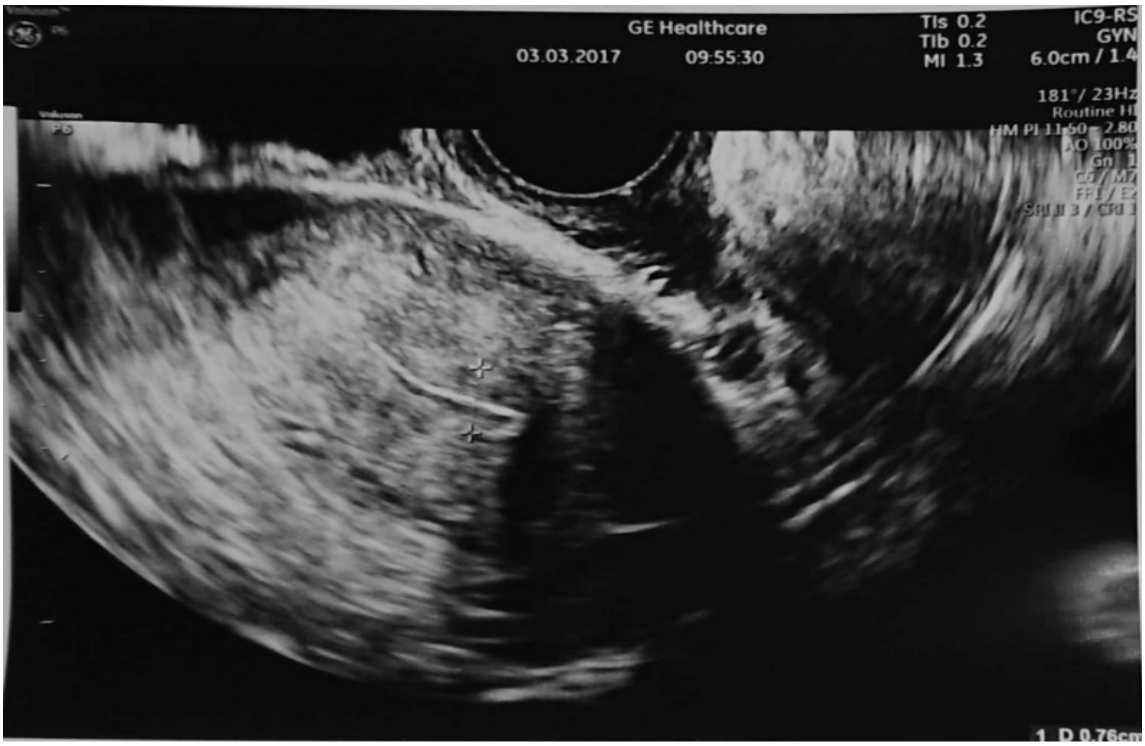


Figure 2. Endometrium of the first patient after MSC administration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Routine
HM PI 11.50 - 2.80
AO 100%
Gn 1
C0 / M7
FF1 / E2
SP1 3 / CP1 1
1 D 0.76cm

Figure 3. Endometrium of the second patient after MSC administration