Brief Report

GLOBAL Reproductive HEALTH

Use of autologous adipose-derived mesenchymal stem cells for ovarian rejuvenation in poor responder IVF patients: a phase 1 randomized placebo-controlled double-blind crossover study

Mohamed Iqbal Cassim, MD[a,b], Tasneem Mohamed, MD[a,*], Jamila Kathoon Adam, PhD[b], Carola Niesler, PhD[c,d], Admire Chikandiwa, MD, PhD[e]

Background: Despite the application of various methods to augment ovarian responsiveness, the management of poor ovarian responders remains challenging and pregnancy rates following in vitro fertilization are poor. Advances in adult stem cell research and their clinical application has prompted interest in their use in assisted reproduction. We report the first double-blind, randomized, placebo-controlled clinical study using autologous human stromal vascular fraction (SVF) containing adipose-derived stem cells (ADSCs) for ovarian rejuvenation.

Materials and methods: Thirty patients were recruited. Twenty-one had lower-than-expected reserves for their age and 9 had premature ovarian insufficiency. Patients were randomized into a placebo group (10) and an intervention group (20). SVF was obtained from adipose tissue following abdominal liposuction; the ADSC component was characterized using flow cytometry. Three equal insertions, adjusted based on ovarian volume, were performed at monthly intervals via an ultrasound-guided transvaginal needle puncture. The SVF was not cultured before transplantation. Those in the placebo group were then crossed over to the intervention group and received a single SVF (maximally concentrated) insertion (crossover group).

Results: The median viable SVF cell number inserted per patient over 3 months, and the percentage of mesenchymal stem cells (MSC) thereof, was 1.6×10^6 and 13.2%, respectively. Resulting anti-Mullerian hormone (AMH) changes were variable over the treatment course with a notable placebo effect. Patients with premature ovarian insufficiency showed no change in AMH, both to intervention and placebo. Despite this, a temporary return of menses was noted in a third of patients while on treatment. Patients with low reserves for age showed an increase in AMH, although not statistically significant when compared to placebo. In the crossover group, insertions were limited to one intervention comprising all cells; here a significantly higher median of 3.4×10^6 SVF cells were injected containing an average of 16.9% MSCs. No significant change in AMH was noted. To date 12 patients have undergone ovarian stimulation and in vitro fertilization after stem cell therapy; of these 9 have had embryo transfers with a resulting pregnancy rate of 33%. There were also 2 spontaneous pregnancies.

Conclusion: Although the application of SVF-derived ADSCs for ovarian rejuvenation remains experimental, the current study provides further support for the safety of this approach and presents encouraging results as to its efficacy in assisted reproduction.

Keywords: Stem cells, Premature ovarian insufficiency, Low ovarian reserves, Ovarian rejuvenation

Introduction

The clinical management of poor responder (PR) in vitro fertilization (IVF) patients remains a challenge for assisted reproductive technology practitioners. The definition of a PR has traditionally taken into consideration a woman's age, biomarkers of her ovarian reserve, and her previous IVF cycle history. According to the European Society of Human Reproduction and Embryology

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

[a]*BioART Fertility Clinic, Johannesburg, South Africa,* [b]*Department of Biomedical and Clinical Technology, Durban University of Technology (DUT), Durban, South Africa,* [c]*Discipline of Biochemistry, School of Life Sciences, University of KwaZulu-Natal (UKZN), Pietermaritzburg, South Africa,* [d]*Next Biosciences, Midrand, South Africa and* [e]*Department of Obstetrics and Gynaecology, University of the Witwatersrand, Johannesburg, South Africa*

**Corresponding author. Address: 99 Oxford Road, Saxonwold, Johannesburg 2196, South Africa. Tel: +27 725355228. Email address: tm.mohamed@yahoo.com (T. Mohamed).*

Global Reproductive Health (2023) 8:e68

Received 26 May 2022; Accepted 4 January 2023

Published online 13 April 2023

http://dx.doi.org/10.1097/GRH.0000000000000068

Table 1

Inclusion and exclusion criteria.

Criteria	Description
Specific exclusion criteria	History of current or previous malignancy
	Chronic medical conditions, previous ovarian or tubal surgery, endometriosis, PCOS
	Any other co-existing cause of infertility in the couple (male or female factors)
	Inability to visualize the ovaries on transvaginal ultrasound
	Smoker
Specific withdrawal criteria	Unwillingness to partake in study or comply with requirements
	Any serious side effect experienced whereby the researchers believes it will be ill-advised to continue with treatment
Specific inclusion criteria	Age <42 y
	Poor ovarian responder (in accordance with ESHRE guidelines)
	Diminished ovarian reserve

ESHRE indicates European Society of Human Reproduction and Embryology; PCOS, polycystic ovary syndrome.

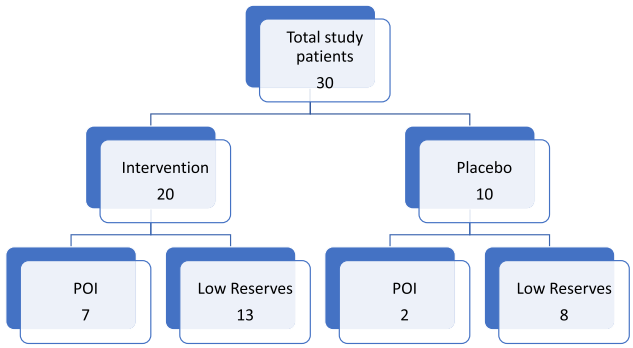

Figure 2. Patient distribution post-randomization. POI indicates premature ovarian insufficiency.

(ESHRE), to be defined as a poor ovarian responder at least 2 of the following 3 features must be present: advanced maternal age ($\geq$40 y), an abnormal ovarian reserve test [antral follicle count (AFC) <5 or anti-Mullerian hormone (AMH) levels <0.5 ng/mL] and/or previous poor response to ovarian stimulation (as reflected by cycle cancelation and/or fewer than 4 oocytes retrieved despite adequate ovarian stimulation)[1]. Despite the existence of various methods to augment the ovarian response to stimulation such as growth hormone[2–6], dehydroepiandrosterone[7–9], and alternate stimulation protocols, pregnancy rates and outcomes in PRs remain low. The alternative of using donor oocytes is often proposed in such cases. However, this is unacceptable to couples who desire only to have a biological child.

Advances in adult stem cell research and its potential clinical application in fertility have prompted interest in the use of stem cells in assisted reproduction, particularly for ovarian and testicular rejuvenation[10], treatment of Asherman syndrome[11–14], endometrial implantation therapy for resistantly thin endometrium and recurrent implantation failure[15]. Favorable results from studies of gametogenesis in rodent models have been greatly encouraging[16–23]. However, the extrapolation of this information to human reproduction poses several challenges. To date, published studies on human ovarian rejuvenation with stem cells[24,25] have been limited by small sample sizes, lack of randomization, and were not placebo controlled.

To address this knowledge gap, we designed a double-blind, randomized, placebo-controlled study to determine if autologous stromal vascular fraction (SVF) containing adipose-derived stem cells (ADSCs), injected into the ovary of women with reduced ovarian reserves or premature ovarian insufficiency (POI), can significantly improve ovarian function, stimulate ovarian responsiveness, and ultimately increase fertility potential. To our knowledge, this is the first such study on humans.

Materials and methods

Study design and subjects

The randomized trial was approved by the Durban University of Technology Institutional Research Ethics Committee (ethics clearance number IREC 042/19) and registered on the Pan African Clinical Trial Registry database, identification number PACTR202007828853091. Written informed consent was obtained from all participants. The study was powered to detect a mean difference of 1.2 in the change in AMH levels between the placebo and treatment group with an 80% power, at a 5% significance level and assuming a randomization ratio of 1:2. The values obtained from the Stata power calculator were 9:18 and

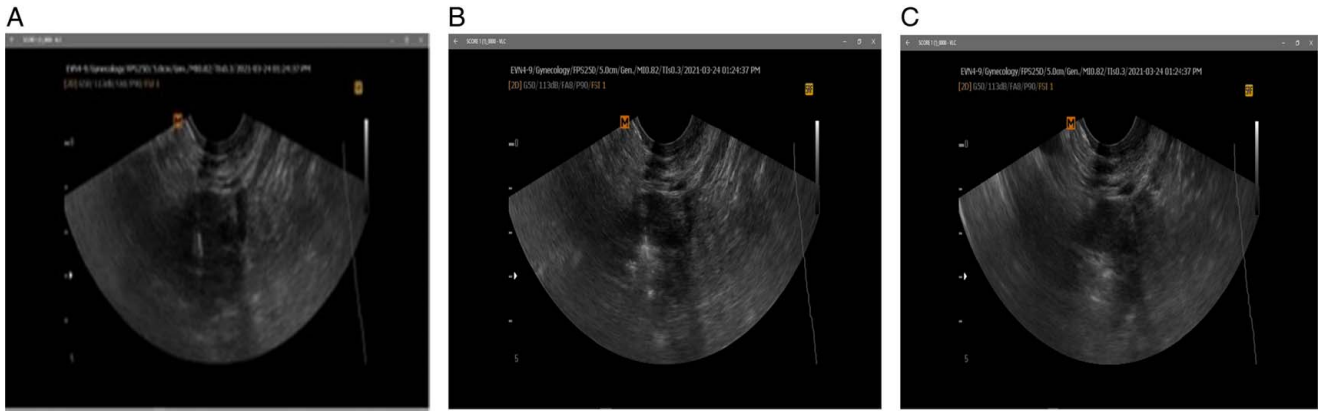

Figure 1. Ultrasound-guided transvaginal needle puncture technique used in the current trial. A, Needle insertion into ovary. B, Injection of stromal vascular fraction. C, Residual opacification after needle withdrawal.

Table 2

Patient characteristics of infertility patients enrolled in the placebo and intervention groups.

	Placebo (N = 10)	Intervention (N = 20)	Total (N = 30)	P*
Age				0.843
Median (IQR)	37.00 (33.00, 42.00)	36.50 (33.50, 39.50)	37.00 (33.00, 40.00)	
BMI				0.333
Median (IQR)	25.40 (22.30, 32.10)	29.00 (24.20, 31.85)	28.20 (23.90, 32.10)	
Right ovarian volume (cm^3)				0.792
Median (IQR)	2.63 (2.01, 3.46)	2.96 (1.96, 4.01)	2.79 (2.01, 3.94)	
Left ovarian volume (cm^3)				0.826
Median (IQR)	2.79 (1.94, 4.19)	3.24 (1.52, 4.04)	3.17 (1.80, 4.19)	
Right uterine artery doppler (RI)				0.495
Median (IQR)	0.93 (0.77, 1.00)	0.99 (0.87, 1.00)	0.99 (0.83, 1.00)	
Left uterine artery doppler (RI)				0.628
Median (IQR)	1.00 (0.88, 1.00)	1.00 (0.87, 1.00)	1.00 (0.87, 1.00)	
Right ovarian artery doppler (RI)				0.809
Median (IQR)	0.96 (0.85, 1.00)	0.97 (0.81, 1.00)	0.97 (0.82, 1.00)	
Left ovarian artery doppler (RI)				0.567
Median (IQR)	0.97 (0.85, 1.00)	1.00 (0.91, 1.00)	1.00 (0.89, 1.00)	
POI/POF	2 (20.00%)	7 (35.00%)	9 (30.00%)	0.675
Poor responder/low reserves	8 (80.00%)	13 (65.00%)	21 (70.00%)	0.675
Stromal vascular fraction (SVF)				
Median viable cells $\times 10^6$ (IQR)†	3.4 (1.1, 6.4)	1.6 (0.81, 2.8)	1.7 (0.84, 3.3)	0.045
Median % MSC fraction (IQR)	16.9 (9, 21.7)	13.2 (9.3, 17.2)	13.5 (9.0, 17.6)	0.030

*P-value based on Kruskall-Wallis test for numerical variables and Fisher exact test for categorical variables, except for SVF comparisons, where a Student *t* test was used.
†Per patient (total of 3 insertions applied over 3 months in "Intervention" group, and a single insertion of all cells in the subsequent crossover "Placebo" group).
BMI indicates body mass index; IQR, interquartile range; MSC, mesenchymal stem cells; POI, premature ovarian insufficiency; POF, prematutre ovarian insufficiency; RI, resistance index.

these were rounded off to 10:20, respectively, thus giving a total sample size of 30.

A total of 30 patients with diminished ovarian reserves were recruited into the study according to specific inclusion and exclusion criteria (**Table 1**). The average AMH level in the study population was ~5 times lower than the average AMH level expected for that age. Twenty-one of these patients had lower-than-expected ovarian reserves for age and nine had POI, defined in accordance with the European Society of Human Reproduction and Embryology (ESHRE) guidelines[26]. To avoid interobserver bias, all screening as well as interventions were performed by the same clinician. All were evaluated for body mass index (BMI). Transvaginal ultrasound was performed to evaluate AFC, ovarian volumes, right and left uterine and ovarian artery perfusion, uterine size, as well as any anatomic pathology of the uterus, endometrial cavity, cervix, and ovaries (Samsung

Table 3

Patient characteristics of infertility patients enrolled in the placebo and intervention groups among patient with low ovarian reserve.

	Placebo (N = 8)	Intervention (N = 13)	Total (N = 21)	P*
Age				0.856
Median (IQR)	37.00 (32.50, 40.00)	36.00 (33.00, 37.00)	37.00 (33.00, 38.00)	
BMI				0.515
Median (IQR)	25.40 (20.95, 29.85)	28.90 (23.80, 31.30)	26.00 (22.80, 31.30)	
Right ovarian volume (cm^3)				0.515
Median (IQR)	2.79 (2.27, 4.63)	3.44 (2.90, 4.10)	3.11 (2.53, 4.10)	
Left ovarian volume (cm^3)				0.664
Median (IQR)	3.48 (2.10, 4.20)	3.71 (3.23, 4.50)	3.71 (2.25, 4.31)	
Right uterine artery doppler (RI)				0.469
Median (IQR)	0.83 (0.76, 1.00)	0.88 (0.83, 1.00)	0.88 (0.80, 1.00)	
Left uterine artery doppler (RI)				0.800
Median (IQR)	1.00 (0.88, 1.00)	1.00 (0.88, 1.00)	1.00 (0.88, 1.00)	
Right ovarian artery doppler (RI)				0.328
Median (IQR)	0.94 (0.81, 1.00)	0.89 (0.76, 0.95)	0.89 (0.77, 1.00)	
Left ovarian artery doppler (RI)				0.365
Median (IQR)	0.97 (0.84, 1.00)	1.00 (0.93, 1.00)	1.00 (0.93, 1.00)	
Stromal vascular fraction (SVF)				
Median viable cells $\times 10^6$ (IQR)†	3.4 (1.3, 6.3)	0.95 (0.8, 2.7)	1.4 (0.8, 3.1)	0.044
Median % MSC fraction (IQR)	14.1 (8.6, 23.3)	11.8 (8.8, 16.1)	11.8 (8.7, 18.0)	0.046

*P-value based on Kruskall-Wallis test for numerical variables and Fisher exact test for categorical variables, except for SVF comparisons, where a Student *t* test was used.
†Per patient (total of 3 insertions applied over 3 months in "Intervention" group, and a single insertion of all cells in the subsequent crossover "Placebo" group).
BMI indicates body mass index; IQR, interquartile range; MSC, mesenchymal stem cells; POI, premature ovarian insufficiency; RI, resistance index.

Table 4

Patient characteristics of infertility patients enrolled in the placebo and intervention groups among patient with premature ovarian insufficiency.

	Placebo (N = 2)	Intervention (N = 7)	Total (N = 9)	P*
Age				0.884
Median (IQR)	38.00 (34.00, 42.00)	38.00 (35.00, 41.00)	38.00 (35.00, 41.00)	
BMI				0.884
Median (IQR)	31.20 (23.90, 38.50)	30.90 (27.60, 36.20)	30.90 (27.60, 36.20)	
Right ovarian volume (cm^3)				0.380
Median (IQR)	1.32 (0.60, 2.04)	2.10 (1.32, 2.27)	2.04 (1.32, 2.21)	
Left ovarian volume (cm^3)				0.770
Median (IQR)	2.04 (1.61, 2.46)	1.70 (0.41, 2.59)	1.70 (1.32, 2.46)	
Right uterine artery doppler (RI)				0.770
Median (IQR)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	
Left uterine artery doppler (RI)				0.242
Median (IQR)	1.00 (1.00, 1.00)	0.88 (0.85, 1.00)	1.00 (0.87, 1.00)	
Right ovarian artery doppler (RI)				0.380
Median (IQR)	0.96 (0.91, 1.00)	1.00 (1.00, 1.00)	1.00 (1.00, 1.00)	
Left ovarian artery doppler (RI)				0.770
Median (IQR)	0.96 (0.91, 1.00)	1.00 (0.81, 1.00)	1.00 (0.86, 1.00)	
Stromal vascular fraction (SVF)				
Median viable cells × 10^6 (IQR)†	3.5 (1.9, 5)	2.5 (1.9, 3.0)	2.5 (1.6, 3.4)	0.631
Median % MSC fraction (IQR)	16.9 (16.6, 17.2)	17.1 (13.0, 17.6)	17.1 (13.5, 17.6)	0.522

*P-value based on Kruskall-Wallis test for numerical variables and Fisher exact test for categorical variables, except for SVF comparisons, where a Student *t* test was used.
†Per patient (total of 3 insertions applied over 3 months in "Intervention" group, and a single insertion of all cells in the subsequent crossover "Placebo" group).
BMI indicates body mass index; IQR, interquartile range; MSC, mesenchymal stem cells; RI, resistance index.

Medison-R7-EVN4-7 Transvaginal probe). Baseline hormonal assay included AMH (Beckman Coulter), FSH, LH, estradiol, progesterone, thyroid function, and prolactin (Lancet Laboratories, Ampath Laboratories). All patients were further screened for HIV and had routine cervical cytological evaluation if not done in the preceding 12 months. Abdominal liposuctions, SVF isolation, and insertions were all carried out between the period of December 2020 and October 2021.

SVF isolation

Adipose tissue (40–225 mL) was obtained by abdominal liposuction under local anesthesia. All liposuctions were uncomplicated. To extract the SVF containing ADSCs, the lipo-aspirate was processed by Next Biosciences (Midrand, South Africa) within 30 hours of the procedure. Stem cell extraction took place in a sterile clinical grade 8 clean room. Adipose tissue was enzymatically digested using collagenase NB6 GMP-grade enzyme (Nordmark; N0002779), after which the isolated cells were thoroughly washed by centrifugation in sterile saline solution and strained through a sterile 100 μm filter. Sterility testing of all samples (5 d aerobic/anaerobic growth monitoring) was conducted using the standardized BacT/Alert System (bioMerieux). The SVF was then cryopreserved until required in 1 mL aliquots using a CTS Synth-a-freeze cryopreservation medium (Life Technologies; A1371301) and liquid nitrogen in a controlled-rate freezer.

Flow cytometry

The SVF contains a heterogenous mixture of cells, a portion of which are stem cells. Flow cytometry testing was therefore conducted on each sample to determine the exact number of viable mesenchymal stem cells present using the DURAClone SC Mesenchymal Stem Cell Antibody Panel (Beckman Coulter; C34369) and ViaKrome 638 viability dye (Beckman Coulter; C36624). Samples were acquired using a Beckman DxFLEX clinical flow cytometer. ADSCs were distinguished as negative for CD14, CD19, CD31, and CD45, while positive for CD34, CD73, CD90, and CD105, as per published criteria[30].

Table 5

AMH changes among premature ovarian insufficiency patients in the placebo and intervention groups.

	Placebo (n = 10)	Intervention (n = 20)	Total (n = 30)	P*
Baseline AMH (μmol/L)				0.281
Median (IQR)	0.41 (0.09, 0.75)	0.17 (0.03, 0.58)	0.22 (0.03, 0.60)	
Maximum AMH (μmol/L)				0.582
Median (IQR)	0.28 (0.18, 1.09)	0.38 (0.03, 0.69)	0.33 (0.03, 0.91)	
AMH expansion (baseline to max)				0.262
Median (IQR)	0.24 (0.00, 0.54)	0.05 (0.00, 0.20)	0.09 (0.00, 0.21)	

*P-value based on Kruskall-Wallis test for numerical variables.
AMH indicates anti-Mullerian hormone; IQR, interquartile range.

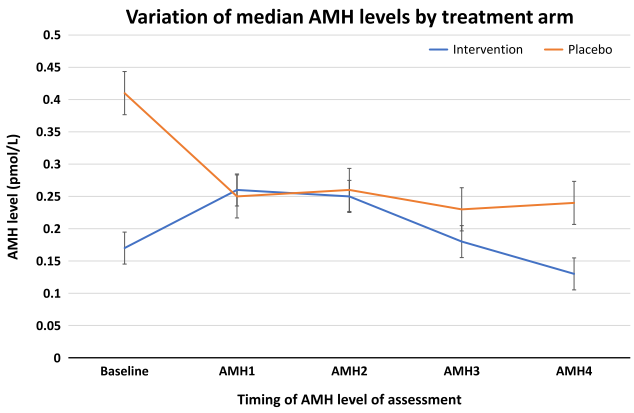

Figure 3. Anti-Mullerian hormone (AMH) levels in placebo and intervention groups. AMH was measured prior to the first insertion (baseline) and then subsequently 2 weeks following each insertion (AMH1, AMH2, AMH3). A final AMH was measured 2 months after the last insertion (AMH4).

SVF release and insertion procedure

When requested, the appropriate SVF sample vial was rapidly thawed at 37 °C, washed 3 times with phosphate-buffered saline (PBS), and resuspended to a clinician-prescribed volume. All procedures were carried out under sterile conditions. The SVF and placebo fluid (PBS), were randomly assigned by the Next Biosciences team so that the clinician was blinded as to what was being inserted. The volume of fluid to be inserted was proportioned to 40% of the predetermined volume of each ovary to avoid undue distension of the ovary as well as retrograde spill post-insertion. All insertions were performed under conscious sedation with ultrasound-guided transvaginal needle puncture using a 35 cm 18-G single-lumen ovum aspiration needle (Cook). The insertion technique aimed to position the needle tip 3–4 mm into the ovarian cortex, checking for inadvertent vascular penetration with color flow doppler, steady flush to observe the pattern of opacification and finally, observing for localized persistent opacification after needle withdrawal (**Fig. 1**). Insertions into very small volume ovaries (below 1.0 cm^3) proved technically difficult, with respect to stabilization/immobilization of the ovary before injection. Insertions were performed at monthly intervals for 3 months. All insertions were uncomplicated except for 1 patient who developed a mild pelvic infection after 1 of the insertions which responded rapidly to antibiotic therapy. Serum AMH was measured 2 weeks after each insertion and a final AMH was measured 2 months after the final insertion.

Patient follow-up

Patients were followed up monthly during the trial, and then 2 months after the last insertion. Participants who had been randomized to the placebo group were then consulted to discuss the preliminary results of the study. These individuals were then offered a single SVF insertion of all cryopreserved cells. At each visit, an ultrasound was done before the insertion to assess AFC. Patients were required to report any adverse effects or critical incidents, as well as any medication use history since the previous treatment.

Statistical analysis

The data were imported to Stata 15.0 (StataCorp) for data analysis. Categorical variables were described using frequencies and percentages, while continuous variables were described using medians [with interquartile range (IQR)] as the data were non-parametric. Categorical variables were compared using the Fisher exact test as the cell sizes were small. Continuous variables were compared using the Kruskall-Wallis test. Correlations between variables were assessed using Pearson correlation coefficients. Differences in SVF numbers were assessed using a standard Student *t* test. A *P*-value of <0.05 was considered to be statistically significant.

Results

Baseline characteristics of the study population

Of the 30 enrolled study patients, 10 were assigned to the placebo group and 20 to the intervention group (**Fig. 2**). The median (IQR) age and BMI were 37 (33–40) and 28.2 (23.9–32.1), respectively. No statistically significant differences were noted between the 2 groups when matched for age, BMI, ovarian volumes, and ovarian and uterine arterial perfusion, that us, resistance index (RI) (**Table 2**). The median (IQR) viable SVF cell number isolated for all participants and the % MSC thereof was 1.7×10^6 ($0.84–3.3\times10^6$) and 13.5% (9.0%–17.6%), respectively. These values were significantly different when comparing the 2 groups ($P<0.05$; **Table 2**). The placebo group recorded median (IQR) values of 3.4×10^6 ($1.1–6.4\times10^6$) and 16.9% (9%–21.7%) while the intervention group values were 1.6×10^6 ($0.81–2.8\times10^6$) and 13.2% (9.3%–17.2%).

Twenty-one patients (70%) had lower than expected ovarian reserves for age (**Table 3**), while 9 (30%) had POI (**Table 4**). Post-randomization, there were 8 patients with low reserves in the placebo group and 13 in the intervention group, while 2 patients with POI were in the placebo group and 7 were in the intervention

Table 6

AMH changes among patients enrolled in the placebo and intervention groups with low ovarian reserves.

	Placebo (n = 8)	Intervention (n = 13)	Total (n = 21)	*P**
Baseline AMH				0.664
Median (IQR)	0.53 (0.28, 0.77)	0.49 (0.17, 0.63)	0.49 (0.21, 0.63)	
AMH max				0.913
Median (IQR)	0.69 (0.26, 1.12)	0.58 (0.38, 0.91)	0.58 (0.29, 1.08)	
AMH expansion (baseline-max)				0.138
Median (IQR)	0.38 (0.08, 0.73)	0.18 (0.07, 0.21)	0.19 (0.07, 0.34)	

**P*-value based on Kruskall-Wallis test for numerical variables.
AMH indicates anti-Mullerian hormone; IQR, interquartile range.

Table 7

AMH changes among placebo crossover group.

	Placebo control (n = 10)
Baseline AMH	
Median (IQR)	0.41 (0.09, 0.75)
Cross over AMH	
Median (IQR)	0.27 (0.10, 0.92)
Baseline to crossover expansion	
Median (IQR)	− 0.01 (− 0.24, 0.14)

Sign test for equality of the baseline and crossover medians showed no statistically significant difference at $\alpha = 0.05\%$ (P-value = 1.000).

Wilcoxon test of equality of the baseline and crossover AMH showed no statistically significant difference at $\alpha = 0.05\%$ (P-value = 1.000).

AMH indicates anti-Mullerian hormone; IQR, interquartile range.

group (**Fig. 2**). Baseline characteristics of the patients were similar between the placebo and intervention groups even when the analysis was limited to those with low ovarian reserves and POI. However, when analyzing viable SVF cell numbers, the values were significantly different between the placebo and intervention group in the low ovarian reserve, but not the POI, cohort (**Tables 3, 4**). The median (IQR) viable SVF cell number isolated for low ovarian reserve participants and the % MSC thereof was 1.4×10^6 ($0.8–3.1 \times 10^6$) and 11.8% (8.7%–18%), respectively. The placebo group in the low ovarian reserve arm recorded median (IQR) values of 3.4×10^6 ($1.3–6.3 \times 10^6$) and 14.1% (8.6–23.3%) while the values in the intervention group of the same arm were 0.95×10^6 ($0.8–2.7 \times 10^6$) and 11.8% (8.8%–16.1%) ($P < 0.05$; **Table 3**).

Changes in AMH level

Placebo versus intervention

Median (IQR) baseline AMH in the placebo and intervention groups were similar 0.41 (0.09–0.75) versus 0.17 (0.03–0.58) (**Table 5**; $P = 0.281$). The median maximum AMH in the intervention group (0.28) was nonsignificantly ($P = 0.582$) lower than that of the control group (0.38). The median (IQR) AMH expansion (ie, baseline to max) was not significantly different between the placebo group at 0.24 (0.00–0.54) and the intervention group at 0.05 (0.00–0.20) (**Table 5**; $P = 0.262$). The greatest expansion in AMH was noted following the first insertion of SVF in the intervention group; but subsequent measurements revealed no significant changes (**Fig. 3**). On the basis of these preliminary findings, the crossover group (patients previously assigned to the placebo group) was subjected to a single stem cell insertion with a substantially higher median SVF cell number of 3.4×10^6 (**Table 2**). We noted no measurable change in the AMH level in the patients with POI (below measurable threshold of 0.03 ng/mL). There were also no significant differences in baseline AMH, maximum AMH, and baseline to maximum AMH when the analysis was limited to patients with low ovarian reserves (**Table 6**).

Crossover group

As mentioned previously, all patients originally randomized to the placebo group (**Table 2**) were then offered a single insertion of all SVF cells and AMH levels were determined. The median (IQR) baseline and cross-over AMH were 0.41 (0.09–0.75) and 0.27 (0.10–0.92), respectively. The median (IQR) AMH baseline to crossover expansion analysis suggests a slight decline in AMH levels [− 0.01(− 0.24, 0.14)] (**Table 7**). More importantly, there was no significant difference between the baseline and cross-over median AMH levels ($P = 1.000$).

Return to menses

Feedback from 9 patients with POI (7 from the initial intervention cohort and 2 from the crossover cohort; **Table 8**) revealed that the majority (55.6%) experienced some return to regular menstruation during the trial.

Correlations between important variables

Volume fat, stem cell count, and the total number of viable cells

There were significant positive correlations between the volume of fat and total viable cells isolated (Pearson coefficient = 0.6390; $P = 0.0001$) as well as total viable ADSCs (ADSC or MSC as determined by flow cytometry; Pearson coefficient = 0.632, $P = 0.002$), suggesting that higher volumes yield not only more cells, but positively impact the viability of the isolated cells (**Fig. 4**).

BMI, total stem cell count, total viable cell count, and MSC

A significant positive correlation was also noted between BMI and total viable cell count (Pearson coefficient = 0.5122, $P = 0.005$) as well the total viable MSC count (Pearson coefficient = 0.4235, P-value = 0.022). Interestingly, there was no correlation between BMI and % MSC in the SVF fraction (Pearson coefficient = 0.2696, $P = 0.15$), suggesting that although a higher BMI may predict better cell viability, it does not necessarily lead to increase stem cell yield (**Fig. 5**).

Ovarian blood supply, ovarian volume, and AMH response

When analyzing correlations for ovarian blood supply, lower ovarian volumes were associated with statistically significantly higher RI (Pearson coefficient 0.4516, $P = 0.0123$). However, there was no significant correlation between mean ovarian perfusion and AMH response (Pearson coefficient = 0.014, $P = 0.94$) (**Fig. 6**).

Table 8

Menstrual history of patients with premature ovarian insufficiency enrolled in the trial.

Patient #	Intervention/ crossover	Menstrual history
5	Intervention	Irregular menstruation before, regular during the trial, then irregular again
8	Intervention	Irregular menstruation before, nil during trial, twice after the trial, then stopped
9	Intervention	Nil prior, menstruated while on trial, nil after
10	Intervention	Nil prior, during or after
15	Intervention	Nil prior, menstruated while on trial, nil after
16	Intervention	Irregular menstruation before, menstruated for months while on trial and 7 mo post, then stopped
20	Intervention	Nil prior, during or after
25	Crossover	Nil prior, menstruated while on trial and 1 mo post, then stopped
29	Crossover	Nil prior, during or after

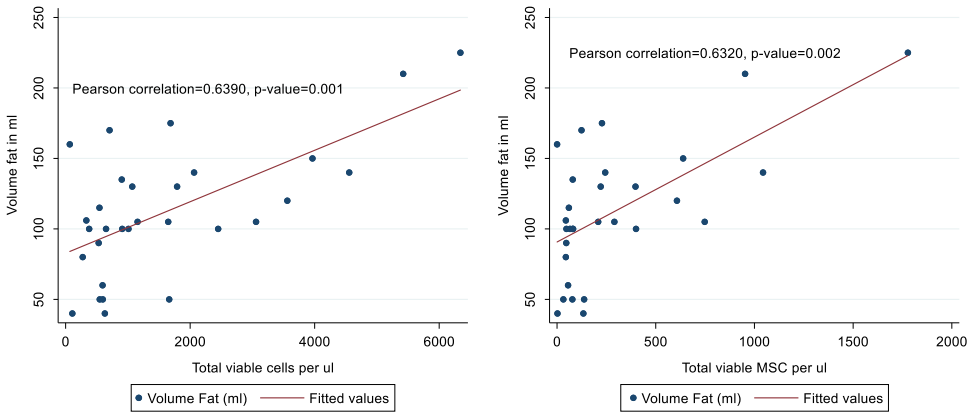

Figure 4. Correlation between volume of fat and total viable cell count and between volume of fat and total number of viable mesenchymal stem cells (MSC).

Clinical outcomes

Thus far, 12 patients have undergone IVF stimulation cycles with an average of 3 oocytes per patient and good embryo quality. One patient for fertility preservation did not have embryo transfer. She had 4 oocytes from a pretreatment cycle and now has a further 8 cryopreserved oocytes from this cycle. Two patient's cycles were abandoned due to poor response. Of the remaining 9 who had embryo transfer, there were 3 pregnancies (33.3% overall pregnancy rate). Overall it is evident that we had more oocytes and more embryos following therapy (**Table 9**). Of the 3 who conceived, 2 were from the crossover group having received a placebo first and then a single insertion of stem cells thereafter (**Table 10**). We also report 2 spontaneous pregnancies in the study population. Both of these were also initially in the placebo group and then crossed over to the treatment arm, and hence had a single stem cell insertion (**Table 10**).

Discussion

As mentioned previously, recent studies and publications on human ovarian rejuvenation with stem cells have shown promising results. However, these studies have been small, lacked randomization and were not placebo-controlled. We opted to use SVF-containing adipose-derived mesenchymal stem cell as adipose tissue is more accessible, and may represent a population with greater yield and mesenchymal potency than the bone marrow-derived stem cells, which are theoretically primed to differentiate along the hematopoietic lineages. adipose-derived mesenchymal stem cell, on the other hand, have been shown to differentiate along multiple mesodermal lineages. We aimed to obtain a minimum of 100 mL subcutaneous fat aspirate. In subjects with low BMI, abdominal liposuction proved to be somewhat challenging, with smaller volumes of aspirate obtained. It was subsequently noted that the volume of adipose aspirate correlated well with the total mesenchymal stem cell and total viable SVF cell numbers.

Subanalysis of those with low reserves versus those with POI in both the treatment and the placebo group also showed that they were matched and hence the success of the randomization algorithm. In the initial intervention group, where 20 participants received 3 equal volumes of SVF over 3 months, the most favorable response occurred after insertion 1 in the intervention arm, which is in keeping with other studies that had only a single treatment. The approach of the current trial was to standardize the SVF insertion to the volume of each ovary and administer the maximum number of cells for each participant. The number of

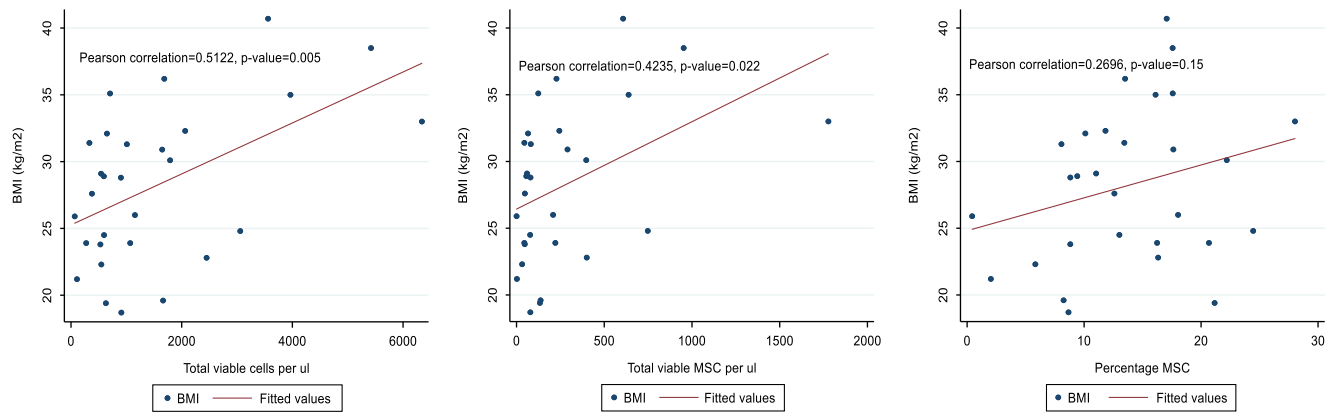

Figure 5. Correlations between BMI and total viable cell count, total viable MSC count or % MSCs. BMI indicates body mass index; MSC, mesenchymal stem cells.

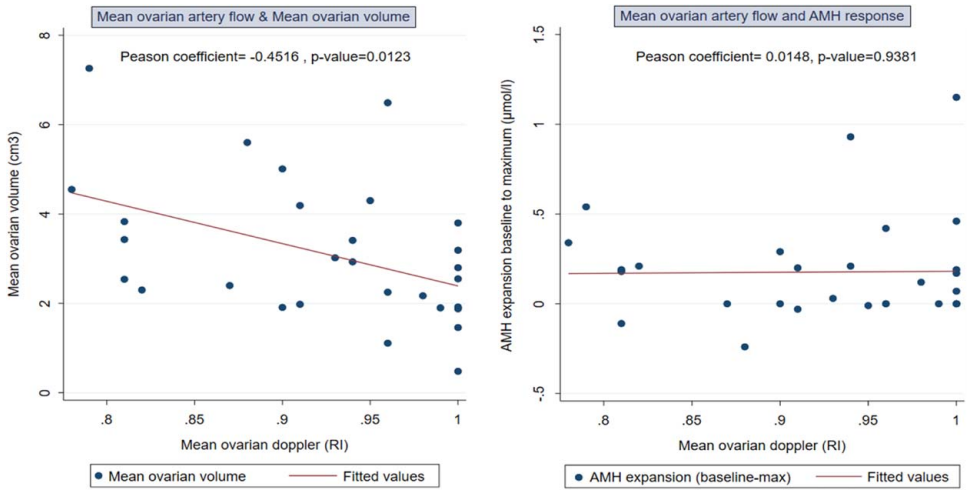

Figure 6. Correlation between ovarian blood supply and either ovarian volume or anti-Mullerian hormone (AMH) response.

SVF cells isolated (and % MSC thereof) was therefore variable; interestingly instances of success were not associated with the highest cell number.

POI patients with AMH levels below 0.03 ng/mL showed no measurable change in AMH levels, both to intervention and placebo. This may in part be due to the threshold sensitivity of the AMH test not being able to measure any changes at extremely low levels. Despite this we did, however, note an increase in AFC in some of the subjects within this group, and few reported having resumed menstruating following treatment. Such findings have also been mirrored in other studies. Patients with low ovarian reserves showed an increase in AMH levels, but this was not statistically significant when compared with placebo. In the crossover group, we limited insertions to one intervention only, based on the preliminary data obtained from the study which showed that maximal expansion in AMH was noted following the first insertion. However, we still failed to show any significant changes in AMH. This may, however, be consequent to a small sample size.

Previous studies have varied in terms of the source of stem cells utilized as well as in the methods of insertion. Two studies have looked at bone marrow-derived hematopoietic stem cells. In one study these stem cells were infused laparoscopically unilaterally into the ovary[27] and in another study, insertion was by radiologically guided catheterization into the ovarian artery[24]. Another study used adipose-derived mesenchymal stem cell with transvaginal insertion via ultrasound guidance into the ovary in 7 cases and laparoscopically in 2 cases[25]. The rationale for arterial infusion remains obscure, based on the likely rapid dissipation of the stem cells into the systemic circulation. The fact that the ipsilateral ovary benefits from the infusion suggests a possible paracrine or even systemic effect. The postulated theory of a local apocrine effect needs further validation.

The choice of stem cell source remains debatable. Whether ADSCs may be advantageous over bone marrow-derived stem cells, or whether there is an even better source such as skin-derived stem cells or very small embryonic-like stem cells needs to be determined from further study. Research into specific cell

Table 9

Key characteristics of the patients who underwent an IVF cycle following stem cell therapy.

	Age		Oocyte no		Fertilization rate		Embryo no.		Embryo grade	
Patient #	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
4	31	33	5	8	60%	100%	3	8	8 II, 2 II, 2 II	1 BB, CMx6, 5 IV
5	33	35	0	1	NA	100%	NA	1	NA	4 II
11	**25**	**26**	**4**	**5**	**100%**	**80%**	**4**	**4**	**8 I, 5 IV, 5 IV, 4 II**	**7 II, 8 II, 8 II, 4 IV**
12	42	42	1	1	0%	0%	NA	NA	NA	NA
13	**41**	**42**	**4**	**3**	**100%**	**100%**	**4**	**3**	**7 II, 6 II, 8 II, 5 II**	**8 I, 7II, 5 II**
14	41	42	0	1	NA	100%	NA	1	NA	3 IV (no transfer)
16	38	41	0	0	NA	NA	NA	NA	NA	NA
18	33	34	1	1	100%	0%	1	0	4 IV	NA
20	NA	28	NA	0	NA	NA	NA	NA	NA	NA
23	**36**	**39**	**1**	**4**	**100%**	**50%**	**1**	**2**	**5 II**	**8 II, 8 II**
26	34	35	4	8	NA	NA	NA	NA	NA	NA
30	36	39	1	2	100%	100%	1	2	6 II	8 II, 6 II
Total			21	34			14	21		

Patients indicated in bold subsequently conceived following IVF.

NA indicates not applicable; Pre: IVF cycle prior to cell therapy; Post: IVF cycle after cell therapy.

Table 10

Key characteristics of the patients who conceived through IVF (A) or naturally (B) following stem cell therapy.

Patient #	Age	Previous failed IVF cycles	Initial treatment or cross over	No. oocytes	No. embryos	Grade of transferred embryos	Total viable cells/mL	%MSC	BMI	Volume of fat (mL)
A										
11	27	1	Crossover	5	4	8II, 8II, 7II	3.25×10^5	10.1	32.1	100
13	42	1	Treatment	3	3	8I, 7II, 5II (day 2)	2.98×10^5	13.0	24.5	50
23	39	2	Crossover	4	2	8II, 8II	4.55×10^5	8.7	18.7	100
Patient #	Age	Previous failed IVF cycles	Initial treatment or cross over	Total viable cells/mL	%MSC	BMI	Volume of fat (mL)			
B										
4	33	1	Crossover	1.66×10^6	8.2	19.6	50			
26	37	0	Crossover	2.19×10^5	5.8	22.3	50			

BMI indicates body mass index; IVF, in vitro fertilization; MSC, mesenchymal stem cells.

markers as well as the release and effect of cellular products such as exosomes is ongoing. The feasibility of in vitro culturing of such stem cells as an option to increase yield in the future remains to be seen[28,29]. The exact mechanism whereby stem cell therapy may work is uncertain; specifically, whether stem cells contribute to new cells through differentiation or act to rejuvenate or reactivate the endogenous tissue stem cell population in which they are grafted remains a topic of debate. In the ovary, they may stimulate angiogenesis and activate tissue kinins which, in turn, may activate latent endogenous ovarian stem cells via the paracrine effect. This may well explain the positive response observed within the placebo group. While the mechanism of action in the rodent model has been extensively studied, there remains uncertainty about which specific factors dominate, as well as their interdependence upon each other[30]. A compounding factor would be local trauma, which is known to stimulate inflammation, angiogenesis and possible activation of tissue kinins and/or latent endogenous stem cells via the paracrine effect may explain the positive response observed with the placebo group.

Finally, our study raises the question as to whether the current markers of ovarian reserve (AMH/FSH/AFC) are as reliable as once thought, or sensitive enough in the case of stem cell rejuvenation. AMH has been revered as the most preferred serum biomarker of ovarian reserve; however, little is known about the endogenous and exogenous factors which may influence its serum levels. From our data, the AMH response was extremely variable, as has been noted in other studies. In addition, studies, including ours, showed changes in ovarian volume, AFC, and FSH levels; clinically with the resumption of menses, improved ovarian response to ovarian stimulation and pregnancies are reported. Hence, we may need to consider the clinical response to stimulation rather than the AMH serum biomarker alone to determine success until more sensitive/accurate markers are available.

Conclusion

Although the use of stem cells for ovarian rejuvenation in humans remains experimental, our results suggest a positive role in ovarian rejuvenation. The exact mechanism of action, the source of stem cell, stem cell numbers, and concentration as well as the mode of insertion needs further research.

Ethics approval and consent to participate

Approval to conduct this study was sought and granted by the Durban University of Technology Institutional Research Ethics Committee (Ethical Clearance number IREC 042/19). Informed written consent was obtained from all participants. No identifying data was captured. All participants were given a study number, and this was used for all correspondence between the clinic and Next Biosciences.

Consent for publication

Written consent for publication was obtained from each participant on enrolment into the study.

Availability of data and material

Data can be made available by the authors if within reason and upon written request.

Sources of funding

Collaborator Next Biosciences agreed to provide the services of SVF harvesting, processing, and storage free of charge as well as the provision of flow cytometry. BioArt fertility center also provided the service of liposuction, and the insertion free of charge.

Author contributions

M.I.C. conceived and designed the study. He was involved in the conduction of the study as well as the final write-up of the manuscript. T.M. was involved in the protocol and final write-up and also in the conduction of the study. J.K.A. was the main study supervisor, reviewer of protocol, and final write-up. C.N. was involved with the collaboration with Next Biosciences, the randomization and blinding processes as well as write-up regarding the fat processing and SVF protocol, flow cytometry, in the materials and methods section. C.N. also contributed to the final write-up. A.C. was involved with the statistical analysis as well as in the final write-up of the manuscript.

Conflict of interest disclosures

C.N. acts as the Chief Scientific Officer at Next Biosciences. The SVF isolation, cryopreservation, and flow cytometry analysis were carried out (free of charge) by Next Biosciences. The remaining authors declare that they have no financial conflict of interest with regard to the content of this report.

Acknowledgments

The authors acknowledge the following: Next Biosciences for the provision of SVF processing and flow cytometry services at no cost; specifically, Ashleigh Welsh, Jodie Thomas, Daughné Jeffries and Kgaogelo Mphahlela. Voula Stivaktas, from Beckman Coulter, for her guidance during the mesenchymal stem cell flow cytometry analysis of SVF samples. The BioART team for all their work and assistance throughout the study process. Frando Lin for his assistance with statistical analysis. Dr Yusuf Dasoo and Prof Yasmin Adam for their support and input.

Annexure 1

Summary of Next Biosciences Adipose-derived Stromal Vascular Fraction (SVF) Processing Protocol

Summary of process

- Wash adipose tissue with saline solution to remove residual blood and any potential contamination.
- Incubate adipose tissue with collagenase enzyme at 37°C.
- Dilute out collagenase by centrifugation and addition of cold PBS.
- Sterile filter the resultant SVF using a cell strainer.
- Centrifuge the resultant solution to obtain your SVF cell pellet.
- Resuspend SVF in PBS, ensuring an even cell suspension, aspirate 20 µL for cell counting.
- Centrifuge the final SVF, resuspend in cold cryopreservation medium, ensure even cell suspension.
- Cool samples to – 180°C, immediately store in liquid nitrogen.

References

[1] Ferraretti AP, Gianaroli L. The Bologna criteria for the definition of poor ovarian responders: is there a need for revision. Hum Reprod 2014;29: 1842–5.
[2] de Ziegler D, Streuli I, Meldrum DR, *et al*. The value of growth hormone supplements in ART for poor ovarian responders. Fertil Steril 2011;96: 1069–76.
[3] Kolibianakis EM, Venetis CA, Diedrich K, *et al*. Addition of growth hormone to gonadotrophins in ovarian stimulation of poor responders treated by in-vitro fertilization: a systematic review and meta-analysis. Hum Reprod Update 2009;15:613–22.
[4] Weall BM, Al-Samerria S, Conceicao J, *et al*. A direct action for GH in improvement of oocyte quality in poor-responder patients. Reproduction 2015;149:147–54.
[5] Bassiouny YA, Dakhly DMR, Bayoumi YA, *et al*. Does the addition of growth hormone to the in vitro fertilization/intracytoplasmic sperm injection antagonist protocol improve outcomes in poor responders? A randomized, controlled trial. Fertil Steril 2016;105: 697–702.
[6] Yu X, Ruan J, He LP, *et al*. Efficacy of growth hormone supplementation with gonadotrophins in vitro fertilization for poor ovarian responders: an updated meta-analysis. Int J Clin Exp Med 2015;8:4954–67.
[7] Chen SN, Tsui KH, Wang PH, *et al*. Dehydroepiandrosterone supplementation improves the outcomes of *in vitro* fertilization cycles in older patients with diminished ovarian reserve. Front Endocrinol (Lausanne) 2019;10:800.
[8] Zhang M, Niu W, Wang Y, *et al*. Dehydroepiandrosterone treatment in women with poor ovarian response undergoing IVF or ICSI: a systematic review and meta-analysis. J Assist Reprod Genet 2016;33: 981–91.
[9] Artini PG, Simi G, Ruggiero M, *et al*. DHEA supplementation improves follicular microenviroment in poor responder patients. Gynecol Endocrinol 2012;28:669–73.
[10] Volarevic V, Bojic S, Nurkovic J, *et al*. Stem cells as new agents for the treatment of infertility: current and future perspectives and challenges. Biomed Res Int 2014;2014:507234.
[11] Singh N, Shekhar B, Mohanty S, *et al*. Autologous bone marrow-derived stem cell therapy for Asherman's syndrome and endometrial atrophy: a 5-year follow-up study. J Hum Reprod Sci 2020;13:31–7.
[12] Zhao Y, Luo Q, Zhang X, *et al*. Clinical efficacy and safety of stem cell-based therapy in treating Asherman syndrome: a system review and meta-analysis. Stem Cells Int 2020;2020:8820538.
[13] Gao Y, Wu G, Xu Y, *et al*. Stem cell-based therapy for Asherman syndrome: promises and challenges. Cell Transplant 2021;30: 9636897211020734.
[14] Zhang Y, Shi L, Lin X, *et al*. Unresponsive thin endometrium caused by Asherman syndrome treated with umbilical cord mesenchymal stem cells on collagen scaffolds: a pilot study. Stem Cell Res Ther 2021;12: 1–19. doi:10.1186/s13287-021-02499-z
[15] Rungsiwiwut R, Virutamasen P, Pruksananonda K. Mesenchymal stem cells for restoring endometrial function: an infertility perspective. Reprod Med Biol 2020;20:13–9.
[16] Zhang D, Liu X, Peng J, *et al*. Potential spermatogenesis recovery with bone marrow mesenchymal stem cells in an azoospermic rat model. Int J Mol Sci 2014;15:13151–65.
[17] Tamadon A, Mehrabani D, Rahmanifar F, *et al*. Induction of spermatogenesis by bone marrow-derived mesenchymal stem cells in busulfan-induced azoospermia in hamster. Int J Stem Cells 2015;8:134–45.
[18] Mehrabani D, Hassanshahi MA, Tamadon A, *et al*. Adipose tissue-derived mesenchymal stem cells repair germinal cells of seminiferous tubules of busulfan-induced azoospermic rats. J Hum Reprod Sci 2015;8: 103–10.
[19] Cakici C, Buyrukcu B, Duruksu G, *et al*. Recovery of fertility in azoospermia rats after injection of adipose-tissue-derived mesenchymal stem cells: the sperm generation. Biomed Res Int 2013;2013: 529589.
[20] Lai D, Wang F, Chen Y, *et al*. Human amniotic fluid stem cells have a potential to recover ovarian function in mice with chemotherapy-induced sterility. BMC Dev Biol 2013;13:34.
[21] Lai D, Wang F, Yao X, *et al*. Human endometrial mesenchymal stem cells restore ovarian function through improving the renewal of germline stem cells in a mouse model of premature ovarian failure. J Transl Med 2015;13:155.
[22] Liu T, Huang Y, Zhang J, *et al*. Transplantation of human menstrual blood stem cells to treat premature ovarian failure in mouse model. Stem Cells Dev 2014;23:1548–57.
[23] Su J, Ding L, Cheng J, *et al*. Transplantation of adipose-derived stem cells combined with collagen scaffolds restores ovarian function in a rat model of premature ovarian insufficiency. Hum Reprod 2016;31: 1075–86.
[24] Herraiz S, Romeu M, Buigues A, *et al*. Autologous stem cell ovarian transplantation to increase reproductive potential in patients who are poor responders. Fertil Steril 2018;110:496–505.e1.
[25] Mashayekhi M, Mirzadeh E, Chekini Z, *et al*. Evaluation of safety, feasibility and efficacy of intra-ovarian transplantation of autologous adipose derived mesenchymal stromal cells in idiopathic premature ovarian failure patients: non-randomized clinical trial, phase I, first in human. J Ovarian Res 2021;14:5.
[26] European Society for Human Reproduction and Embryology (ESHRE) Guideline Group on POI, Webber L, Davies M, *et al*. ESHRE Guideline: management of women with premature ovarian insufficiency. Hum Reprod 2016;31:926–37.
[27] Igboeli P, El Andaloussi A, Sheikh U, *et al*. Intraovarian injection of autologous human mesenchymal stem cells increases estrogen production and reduces menopausal symptoms in women with premature ovarian

failure: two case reports and a review of the literature. J Med Case Rep 2020;14:108.
[28] Bhartiya D, Shaikh A, Anand S, *et al*. Endogenous, very small embryonic-like stem cells: critical review, therapeutic potential and a look ahead. Hum Reprod Update 2016;23:41–76.
[29] Bharti D, Jang SJ, Lee SY, *et al*. In vitro generation of oocyte like cells and their in vivo efficacy: how far we have been succeeded [published correction appears in Cells. 2020 May 20;9(5)]. Cells 2020;9:557.
[30] Rezaie J, Heidarzadeh M, Hassanpour M, *et al*. The angiogenic paracrine potential of mesenchymal stem cells. In: Khalid Ahmed Al-Anazi, editor. Update on Mesenchymal and Induced Pluripotent Stem Cells. IntechOpen; 2019pp. 1–22. doi:10.5772/intechopen.84433