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ORIGINAL PAPER

Evaluation of human bone marrow mesenchymal stem cells in the treatment of non obstructive azoospermia

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Summary ***Background: Non-obstructive azoospermia (NOA) represents an infertility problem that is usually difficult to treat. Such patients usually have testicular biopsy of germ cell aplasia or spermatogenic arrest. In recent decades, Mesenchymal Stem Cells (MSCs) had been studied thoroughly and proved safe and effective regarding their capability for trans-differentiation into different cell types. The aim of this study was to evaluate the effect of MSCs local intratesticular injection in induction of spermatogenesis.***

Patients and method: The current study included 87 infertile non-obstructive azoospermic patients. Clinical assessment and repeated semen analysis with centrifugation were done to confirm azoospermia. Karyotyping and AZF study were done. Some of the patients had previous testicular biopsy proving a lack of sperm in the testes. Single intratesticular injection of purified MSCs suspension was done.

Results: 20.7% of patients showed sperm in their semen after variable period of time. Hormonal profile among treated patients showed significant improvement regardless success of treatment. Also most of the treated patients appreciated the improvement of their sexual function and libido.

Conclusions: Bone marrow derived MSCs could be a new hope and therapeutic modality for treatment of refractory cases of NOA.

KEY WORDS: *Stem cells; Non-obstructive azoospermia (NOA); Spermatogenesis; Semen analysis.*

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INTRODUCTION

About 1% of males and up to 10-15% of infertile men have azoospermia, which is defined as the absence of sperm in the ejaculate upon evaluation of the centrifuged semen. In two thirds of instances, severe spermatogenic dysfunction, also known as *non-obstructive azoospermia* (NOA), is the primary cause (1). Azoospermia can be categorized as either *obstructive* (OA) or NOA (2).

Many medications like antihypertensive medications and antidepressants might cause NOA; all of them have the potential to disrupt spermatogenesis and even cause non-obstructive azoospermia (3).

The majority of occurrences of azoospermia are due to irreversible testicular illnesses that affect spermatogenesis. These conditions are frequently related to inflammatory, genetic, and endocrine issues. If no obvious causes are found, NOA will be considered idiopathic. The affected testes are tiny and swollen (4, 5).

Male infertility can be brought on by a number of chromosomal or genetic disorders, including Klinefelter syndrome, 47(XXY) syndrome, XX male syndrome, and Y-chromosome microdeletions (6).

The foundation for spermatogenesis and male fertility in males is provided by spermatogonial stem cells (SSCs). Throughout the male reproductive life, SSCs can maintain the self-renewal process, differentiate into spermatozoa, and pass on genetic material to the following generation (7).

Stem cell transplantation is one method of treatment for male infertility related to a problem with spermatogenesis. The reason for this is that stem cells are unspecialized cells being capable of self-renewal, regeneration, and cell differentiation. When existing spermatogonial cells are lost or injured, spermatogenesis can be restored by *spermatogonial cells* (SSCs). Therefore, stem cell transplantation represents an effective method for restoring spermatogenesis in individuals with cancer and other spermatogenic disorders (8).

The *Mesenchymal Stem Cells* (MSC) multi-linear differentiation capacity, moderate immunogenicity, and active involvement in tissue repair and regeneration following migration to injured locations account for their widespread use. For clinical usage in cell-based therapeutics, MSCs generally have an advantage over other types of stem cells (9).

One of the primary sources of MSCs is the bone marrow, and although aspirating the bone marrow is the most painful way to isolate MSCs, it is also the most used method for cell therapy (10).

The differentiation of MSCs into the male or female germ cell epithelium can be induced using a specific combination of growth factors, chemicals and genetic modifications. Distinct methods of differentiation induction have been devised to differentiate distinct types of MSCs into male germ cells. Retinoic acid, growth factors, minerals,

co-culture, conditioned medium, magnetic fields, and gene over-expression are a few examples of these (11).
Mild symptoms such as transitory fever, insomnia, nausea, vomiting, or mild changes at the administration site may occur as a side effect of MSCs treatment, necessitating medical supervision and follow-up (12).
As evidenced by improved expression of germ cell markers, a decrease in apoptosis-induced sterility, a reduction in oxidative stress, and an increase in testosterone production, MSCs transplanted into the testes of NOA demonstrated both activation of spermatogenesis and differentiation into germ cells. The inhibition of *antisperm antibodies* (ASA) may also be a function of MSCs (13).
The implanted MSCs produce a variety of growth factors, including the male germ cell potential factors *transforming growth factor beta* (TGF-) and *bone morphogenetic proteins* (BMPs), which stimulate and restore the recipient's cellular function (14).
The grey area about the effect of MSCs local intratesticular injection to induce spermatogenesis has motivated the authors to conduct this study

Subjects and methods

Study design

The current study was conducted following the ethical perspectives of Helsinki Declaration where ethical approval was obtained from the ethical and research committees of both *Benha* and *Al Azhar Universities*. The current study was conducted at the *Urology Department, Benha University and Andrology* outpatient clinic of *International Islamic Institute for Population Studies and Research, Al-Azhar University*. The study was conducted throughout the period from January.2020 till January 2022. The study included 87 subjects with primary infertility, with non-obstructive azoospermia for at least 2 years durations. All cases were volunteers and informed by explaining complete details of the procedure (expected benefits and possible complications). A written consent was obtained from each patient.
Clinical trial registry at: *clinicaltrials.gov/study/NCT02025270*
Every patient underwent a thorough history-taking process, paying particular attention to the marital history (including age of the couple, length of marriage, and previous marriage
Clinical examination, paid particular attention to gigantism, dwarfism, myxedema, Klinefelter syndrome, mongolism... etc. Local examination was performed to check testicular volume, consistency, varicocele, cord anomalies, and vas alterations in order to rule out obstruction or scrotal enlargements, absence as well as congenital malformations of the testis.
Following a 2-7-day period of abstinence, the semen analysis was performed three times in a row in accordance with WHO guidelines to confirm azoospermia.
Follicle stimulating hormone (FSH), luteinizing hormone (LH), and testosterone measurements and testicular biopsy were used to estimate hormonal profiles and to discover histological patterns underlying azoospermia (such as SCOS and phases of spermatogenic arrest).
Karyotyping was done to display chromosomal abnormalities, such as the XX male syndrome and the Klinefelter syndrome 47XXY. Assessing Y-chromosome microdeletions (AZF) was done to identify the loci of spermatogenesis that were affected (A, B, or C).

Instrumentation

- Laminar Air flow Cabinet (*Biological Safety Cabinet*) Class 11 type A/B3.
- Octomax: for magnetic cell selection (*Miltenyi Biotec, Germany*) MACS Multistand.
- Inverted phase – Contrast microscope: for cell examination and counting.
- Light Microscope: for cell counting (*Leitz*).
- Water bath: for sampling preparations and manipulation.
- Humidified CO^2 Incubator with air Jacket: for Cell Stem Cell cultivation, Model MJPX- C 50.
- Refrigerator to keep the media.
- Centrifuge (*Eppendorf*): to centrifuge the sampling for separation, sedimentation.
- Sterile Pasteur pipettes.
- Automatic pipettes (*10-200 UL, Scororex*).
- Sterile tissue culture tubes & dishes.
- Hemocytometer: Improved Neuber.
- Vortex Mixer: for sample mix & resuspending.

Media

- MACS Buffer; Auto MACS Rinsing Solution (*EDTA, Phosphate Buffered Saline*).
- Dulbecco's Phosphate Buffers Saline (*Phenol red, Calcium and Magnesium*).
- HiSep LSM 1077: Phicoll for cell separation (*buffy coat layer*).
- Alcohol 70% for sterilization.

Methods

Under complete aseptic condition, about 60 ml of bone marrow blood were aspirated from iliac bones. This volume is diluted with *phosphate buffer saline* (PBS). The diluted blood sample was gently layered to Ficoll hypaque
Centrifugation was done to separate the buffy coat layer and aspirate this layer that present at the interphase between the plasma and Ficoll carefully. This separated layer is also subjected to magnetic labeling and separation by CD105 microbeads and FCR blocking antibodies by OctaMACS apparatus to get finally pure *Mesenchaymal Stem Cells* (MSC). These cells are finally re-suspended in 1 ml of PBS. A 30 micr. of this volume are subjected to viability testing using trypan blue and counting by improved Neuber hemocytometer. Cell counting for cases ranged (3.7-5.2 mil/cm), viability around 98.4-99.2%. The extracted cells were injected into cortex of both testes of patients under local anesthesia. The patents were followed after 4 months from injection, by semen analysis, hormonal profile (FSH, LH and testosterone) and testicular volume. The follow up was every 2 months for at least one year.

Results

This current study included 87 infertile male patients with a mean age of 35.87 ± 4.22 years in whom the

repeated spermiogram revealed azoospermia. After MSCs injection, 69 (79.3%) patients did not show sperm in their semen while the remaining 18 (20.7%) patients showed different numbers of sperm in their semen.
The mean age of the non-responding group 36.9 ± 7.35 years. Other clinical data and testicular biopsy results were illustrated in Table 1.
In the successful group, the surprising finding is the rate of responders with *Sertoli Cell only Syndrome* (SCOS) (61.1%). The most prominent parameters among responding cases is normal karyotyping and absence of chromosome Y AZF micro deletion (Table 1).
Table 1 demonstrated that there was a statistically significant decrease in the FSH and LH hormone levels as well as significant increase in the testosterone level after MSCs therapy in the responding group more than the non-responding group.
Karyotyping was the only variable showing statistically significant differences between successful and non-responding patients with SCOS (Table 2).
Regarding patients with SCOS, FSH level after treatment showed significant differences between responding and non-responding. Also FSH percentage of level changes after treatment showed statistically significant differences between both responding and non-responding patients. On the other hand, LH levels showed significant differences between SCOS responders and non-responders either before or after treatment. Also, the hormonal level change percentage was significantly different. Moreover, testosterone showed significant differences between both groups regarding level before, after as well as change`s percentage. All successful cases of SCOS have normal karyotyping and absence of AZF microdeletions (Table 2).
Table 3 Showed that FSH level and percentage of changes after treatment showed significant differences between successful vs. failed cases. There was no statistically significant differences among successful vs. failed cases with different levels of spermatogenic arrest as regards the Testesterone and LH hormones although significant difference was noticed within each group before and after MSC injection.

Table 1.
Comparison between successful vs. non responding cases (clinical, testicular biopsy before, genetic background and hormonal level before and after MSCs therapy).

	Failed	Success	P value
No of patients, n (%)	69 (79.3%)	1 (20.7%)	
Age (Mean ± SD)	36.9 ± 7.35	34.83 ± 5.85	0.269
Testicular Size before MSCs therapy			0.227
Normal, n (%)	35	12	
Small, n (%)	34	6	
Testicular Examination before MSCs therapy			0.231
Normal, n (%)	61	17	
Varicocele, n (%)	7	0	
Cryptorchidism, n (%)	1	1	
Testicular biopsy before MSCs therapy			0.084
Primary spermatocyte, n (%)	9	2	
Secondary spermatocyte, n (%)	3	4	
Spermatid, n (%)	2	1	
SCOS, n (%)	55	11	
Karyotyping before MSCs therapy			0.070
Normal	58	18	
Klinefelter	11	0	
Microdeletion before MSCs therapy			0.779
Normal	65	18	
AZF a	1	0	
AZF b	2	0	
AZF c	1		
F S H (Mean ± SD)			
Before MSCs therapy	24.70 ± 10.2	22.07 ± 5.5	0.301
After MSCs therapy	19.68 ± 8.617	11.96 ± 2.97	< 0.001*
	P = 0.016	P < 0.001*	
% of changes	20.32	45.81	< 0.001*
LH (Mean ± SD)			
Before MSCs therapy	10.18 ± 4.75	7.03 ± 2.07	0.008*
After MSCs therapy	8.27 ± 3.679	5.143 ± 1.014	< 0.001*
	P = 0.023*	P < 0.001*	
% of changes	18.76	26.84	0.01*
Testosterone (Mean ± SD)			
Before MSCs therapy	2.367 ± 1.041	3.44 ± 0.66	< 0.001*
After MSCs therapy	3.52 ± 1.30	5.29 ± 0.77	< 0.001*
	P = 0.041	P < 0.001*	
% of changes	32.76	34.97	0.01*

Table 2.
Comparison between successful vs. non responding cases of SCOS (clinical & genetic background) and hormonal levels.

	Failed	Success	P value
No of patients, n (%)	55	11	
Age (Mean ± SD)	36.93 ± 7.45	35.09 ± 7.012	0.752
Testicular Size before MSCs therapy			0.579
Normal, n (%)	30	7	
Small, n (%)	25	4	
Testicular Examination before MSCs therapy			0.245
Normal, n (%)	48	10	
Varicocele, n (%)	6	0	
Cryptorchidism, n (%)	1	1	
Karyotyping before MSCs therapy			< 0.001*
Normal	44	11	
Klinefelter	11	0	
Microdeletion before MSCs therapy			0.214
Normal	52	11	
AZF a	0	0	
AZF b	2	0	
AZF c	1	0	
F S H (Mean ± SD)			
Before MSCs therapy	19.67 ± 8.5	22.2 ± 5.87	0.093
After MSCs therapy	15.2 ± 13.3	12.29 ± 2.25	< 0.007*
	P = 0.012*	P < 0.001*	
% of changes	22.7	44.6	< 0.001*
LH (Mean ± SD)			
Before MSCs therapy	10.18 ± 4.7	6.7 ± 1.6	0.008*
After MSCs therapy	8.28 ± 3.65	4.96 ± 0.96	< 0.001*
	P = 0.003*	P < 0.001*	
% of changes	18.6	26.11	< 0.001*
Testosterone (Mean ± SD)			
Before MSCs therapy	2.35 ± 1.045	3.4 ± 0.818	< 0.003*
After MSCs therapy	3.5 ± 1.30	5.13 ± 0.8	< 0.001*
P = 0.041	P = 0.04*	0.001*	
% of changes	32.9	33.7	< 0.004*

Table 3.
Comparing hormonal levels among successful vs. failed cases with different levels of spermatogenic arrest.

	Failed	Success	P value
No of patients, n (%)	14	7	
Age (Mean ± SD)	36.9 ± 7.12	34.43 ± 3.2	0.3946
F S H (Mean ± SD)			
Before MSCs therapy	19.45 ± 6.5	21.87 ± 4.94	0.403
After MSCs therapy	16.1 ± 4.98	11.37 ± 2.06	0.30
	P = 0.007*	P < 0.001*	
% of changes	17.2	48	< 0.001*
LH (Mean ± SD)			
Before MSCs therapy	7.53 ± 2.86	7.57 ± 2.6	0.098
After MSCs therapy	6.1 ± 1.96	5.5 ± 1.02	0.46
	P = 0.082	P < 0.073	
% of changes	19	31.3	0.0085*
Testosterone (Mean ± SD)			
Before MSCs therapy	3.19 ± 1.13	3.51 ± 0.272	0.38
After MSCs therapy	4.34 ± 1.18	5.26 ± 0.75	0.078
	P = 0.041	P = 0.002*	0.001*
% of changes	36.1	49.8	0.755

Discussion

Aside from the extremely rare occurrences of patients with hypogonadotropic hypogonadism, *Caroppo E. & Colpi G.* (15) came to the conclusion that azoospermia caused by spermatogenic malfunction is an incurable illness.

MSCs, also referred to as fibroblast precursor cells, are non-hematopoietic cells found in the bone marrow. According to standard criteria established by the *International Society for Cellular Therapy* (ISCT), MSCs must be plastic adhering and capable of differentiating into the osteoblast, adipocyte, and chondroblast lineages. Frequently, they have low immunogenicity (16). MSCs use paracrine and immunomodulatory pathways to provide their beneficial effects (17, 18).

In their meta-analysis investigation, *Wang et al.* (12) found no direct evidence that MSC injection had the potential to cause tumours. Additionally, no major safety incidents were noted. Some research (19) have demonstrated that MSCs can trans-differentiate into spermatogenetic cells in the right milieu. BM (*Bone Marrow*)-MSC-transplanted mice produced germ cells in vivo, according to several research (20, 21).

BM-MSCs have the ability to fuse with the local cells in the damaged area or to differentiate or trans-differentiate into multi-lineage cells, produce paracrine substances to entice the local stem cells to take part in tissue regeneration (22).

Pittenger et al. 2019 (23) concluded that MSC infusion treatments had a very good safety profile during the previous 25 years, which was backed up by more than 950 registered MSC clinical trials that were filed with the FDA.

In the present study, a single intratesticular injection of pure MSCs was used to treat patients. Out of 87 patients, 18 (or 20.7%) showed sperm in their semen at various times. In contrast to the successful case report of *Cassim & Mohamed* (24), using local injection with three sessions of intravenous MSC infusion, our investigation used a single intratesticular injection.

Treatment with MSCs had a favourable impact on hormone levels, decreasing FSH and LH levels and raising blood testosterone levels. Improvements in sexual function and libido were reported alongside elevated testosterone levels in the treated subjects from the patient history. On the other hand, there were notable hormone level disparities between responding and non-responder patients, particularly following treatment. Based on the observation of successful cases when the FSH level was double or more than the usual level, the baseline FSH level appeared to be of little use as a predictor of success. All the successful cases had normal karyotyping and no chromosomal abnormalities in the AZF region. This might be seen as a reliable indicator of a successful outcome.

All SCOS cases that are successful have normal karyotyping and no AZF microdeletions, which was true for all cases that were successful despite having differing testicular biopsy results.

The success rate for cases of SCOS was comparable to other non-obstructive azoospermic cases. This is also a strong indicator of MSCs' capacity for homing and trans-differentiation. To summarize, the complete lack of spermatogonia is not a contraindication to MSC therapy.

Although the hormonal profile significantly improved in the case of the classic Klinefelter syndrome, no sperm were found in the seminal fluid despite decreased FSH, LH, and higher testosterone levels. This study's findings align with those of *Baghae et al.* (25).

No problems were noted during aspiration from the BM or during intra testicular injection in any of the instances that were monitored both during and after the MSCs injection.

Based on *Neri's* (26) findings, we avoided using in vitro numerous replications in this study because they could raise the danger of accumulating genetic and epigenetic modifications and having a negative impact on the cell biology and therapeutic characteristics, safety, and efficacy.

According to an experimental investigation in the ram by *Fedder et al.* (27), pathological testicular abnormalities such scar tissue and micro-calcifications were discovered regardless of the sperm retrieval method used. Additionally, blood supply impairment was linked to it.

Furthermore, *Eliveld et al.* (28) concluded that transient hypogonadism, which can persist up to 26 months, is demonstrated by testosterone levels that are below normal. He also noted that some patients' testicular volume had decreased, but only a small number of patients had *erectile dysfunction* (ED). Because melancholy and anxiety may be the root of ED rather than hypogonadism, it was particularly prevalent in the group of men who had negative sperm retrieval results.

Conclusions

Regardless of baseline hormonal levels, local intratesticular injection therapy using human MSCs may offer some hope for patients with refractory NOA.

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