DOI: 10.4081/aiua.2025.14330

ORIGINAL PAPER

Evaluation of carcinogenicity following intratesticular transplantation of autologous bone marrow-derived mesenchymal stem cells in non-obstructive azoospermia patients: A retrospective analysis

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Summary *Background: Non-obstructive azoospermia (NOA) cases may have severely deficient spermatogenesis and inadequate sperm production. Despite increasing clinical investigations emphasizing the safety of mesenchymal stem cell (MSC) therapy in NOA cases, no article has recently reviewed the adverse events and carcinogenicity after transplantation.*

Objective: the study was conducted to evaluate the safety and potential carcinogenic effects of autologous human bone marrow- MSCs implanted into the testes of patients with NOA.

Methods: This retrospective cohort study was conducted throughout the period from January2017 to January 2022, encompassed 100 patients aged 20-40 years with primary infertility, with NOA for at least 2 years duration who had a confirmed diagnosis of NOA, based on two separate semen analyses showing azoospermia with centrifugation, conducted at least three months apart. Patients were submitted to intratesticular transplantation of autologous bone marrow-derived mesenchymal stem cells. Records of all participants were examined to acquire the demographic data of patients, laboratory investigations including β-HCG, ECG, AFP, LDH, and scrotal ultrasound examination.

Results: there is no significant malignancy or cancer occurrence post- MSCs therapy as indicated by the normal level of the tumor marker levels for LDH (183.4 ± 31.5) u/L, AFP (3.16 ± 1.6) ng/ ml, CEA (2.27 ± 1.1) ng/ ml, and β-HCG (0.95 ± 0.2) mIU/mL. Additionally, scrotal ultrasound showed no focal findings in all cases.

Conclusions: there is no carcinogenic effect of mesenchymal stem cells implanted into testes.

KEY WORDS: *Mesenchymal stem cells; Non-obstructive; Azoospermia; Carcinogenic.*

Submitted 6 September 2025; Accepted 20 September 2025

INTRODUCTION

Obstructive azoospermia accounts for 40% of men who present to infertility practices with azoospermia and it is often successfully treated with *intracytoplasmic sperm injection* (ICSI) using surgically collected sperm (1). Men with *non-obstructive azoospermia* (NOA) may have severely deficient spermatogenesis and inadequate sperm production (2). NOA is most frequently characterized by an elevated FSH and small volume testes, and treatment historically involved donor inseminations or adoption.

Mesenchymal stromal/stem cells (MSCs) are multipotent, undifferentiated cells most commonly derived from bone marrow, although they can also be isolated from other tissues such as adipose tissue, umbilical cord, and dental pulp. MSCs are among the best types of stem cells available for clinical cell therapy. They have several advantages over other stem cells, including multilineage differentiation, secretion of anti-inflammatory cytokines and growth factors, ease of isolation and expansion, and lack of ethical issues. MSCs also exhibit immunosuppressive properties (3). Additionally, in azoospermic rats, MSCs could differentiate into germ cells, spermatids, and spermatocytes in the seminiferous tubules. MSCs transplantation is a promising new treatment method proposed for inducing spermatogenesis and treating male infertility (4).

However, the safety and long-term effects of such therapies remain a concern, particularly regarding the risk of tumorigenesis. The potential carcinogenicity of MSC-based therapies is an area of active investigation, as there are concerns about the possibility of stem cells transforming into malignant cells (5). Preclinical studies have shown that MSCs, under certain conditions such as prolonged

culture, genetic instability, or inflammatory microenvironments, may acquire abnormal phenotypes. Additionally, MSCs may support tumor growth indirectly by promoting angiogenesis, suppressing local immune responses, or secreting pro-tumorigenic cytokines.

Serum tumor markers *alpha fetoprotein* (AFP), *beta human chorionic gonadotropin* (bHCG), and *lactate dehydrogenase* (LDH) represent valuable tools for the clinical monitoring of testicular carcinoma (6). They were first introduced into clinical practice in the 1970s and became international standard tools with the worldwide implementation of the immunologically based ELISA measurement technique. According to current guidelines, serum tumor markers are used to assist timely diagnosis of testicular *germ cell tumors* (GCTs), to accurately stage the disease, to assess the prognostic category of metastasized GCTs, to monitor treatment success, and finally to detect relapses during follow-up.

bHCG is a 38 kDa glycoprotein produced by syncytiotrophoblastic giant cells mainly in chorionic carcinoma (7). AFP is a 70 kDa glycoprotein produced by cells of the yolk sac tumor and rarely by embryonal carcinoma. LDH is a glycolytic enzyme that is present in all cells of the human body and that is released from cells upon cell death. Due to its unspecific origin, the clinical usefulness of LDH is less than that of the other two markers (8).

A recent meta-analysis revealed a prevalence rate of LDH in 40-60% of all GCT cases (8). AFP is exclusively found to be elevated in 10-60% of nonseminomatous GCTs. BHCG is elevated in 10-40% of nonseminomas and in 15-20% of seminomas while prevalence rates apparently depend on clinical stages (9). Therefore, the purpose of this study was to evaluate the safety and potential carcinogenic effects of autologous human bone marrow-derived mesenchymal stromal/stem cells implanted into the testes of patients with NOA by monitoring tumor marker levels (LDH, AFP, CEA, β-HCG) and scrotal ultrasound examinations.

Materials and methods

This retrospective cohort study encompassed 100 patients aged 20-40 years with primary infertility, with NOA for at least 2 years duration who had a confirmed diagnosis of NOA, based on two separate semen analyses showing azoospermia with centrifugation, conducted at least three months apart.

Patients were submitted to intratesticular transplantation of autologous bone marrow-derived mesenchymal stem cells.

All cases were volunteers and informed by explaining complete details of the procedure. Consent was obtained from each patient.

The study was conducted following the ethical perspectives of Helsinki Declaration where ethical approval was obtained from the ethical and research committees of *Al Azhar University*. The current study was conducted at the outpatient clinic of *Andrology Unit of International Islamic Institute for Population Studies and Research, Al-Azhar University*.

The study was conducted throughout the period from January 2017 till January 2022. Patients were followed up after 2 years of intratesticular injection of autologous bone marrow-derived MSCs in NOA patients by tumor marker profiles and scrotal ultrasound.

Patients were excluded from the study if they have history of neoplasms, confirmed immune disorders, or a history of mental illness. Those who had undergone previous surgical sperm retrieval methods or had used drugs, such as chemotherapy, testosterone, or antiandrogens, within the past two years were also excluded.

Bone marrow isolation, characterization and injection

Briefly, under complete aseptic condition, about 60 ml of bone marrow blood were aspired 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 MSCs. 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.

Follow up

Records of all participants were examined to acquire the demographic data of patients, laboratory investigations including β-HCG, CEA, AFP, LDH, and scrotal ultrasound examination conducted two years after MSCs therapy.

Statistical analysis

Statistical analysis was done by SPSS v27 (*IBM©, Chicago, IL, USA*). The Shapiro-Wilks test and histograms were used to evaluate the normality of the distribution of data. Quantitative parametric data were presented as mean and *standard deviation* (SD). Qualitative variables were presented as frequency and percentage (%).

Results

Table 1 showed the demographic and clinical characteristics of 100 patients with a mean age of 36.9 ± 7.35 years. Most cases (67%) had normal testicular size, while 33% had small testicles after MSCs therapy. Testicular examination revealed that in the majority of cases treated with MSCs 73% had normal findings, while 21% had varicocele, and 6% had cryptorchidism.

Table 2 showed that there is no significant malignancy detected two years after MSCs therapy as indicated by the normal level of the tumor marker levels for LDH (183.4 ± 31.5) u/L, AFP (3.16 ± 1.6) ng/ ml, CEA (2.27 ± 1.1) ng/ ml, and β-HCG (0.95 ± 0.2) mIU/mL. Additionally, scrotal ultrasound revealed no focal abnormalities in any of the cases, indicating the absence of structural changes such as tumors, focal lesions, or diffuse microlithiasis within the testes.

Table 1.
Demographic and clinical characteristics of the studied patients treated with mesenchymal stem cells.

	All (n = 100)
Age (Mean ± SD)	36.9 ± 7.35
Testicular Size	
Normal, n (%)	67 (67%)
Small, n (%)	33 (33%)
Testicular Examination	
Normal, n (%)	73 (73%)
Varicocele, n (%)	21 (21%)
Cryptorchidism, n (%)	6 (6%)

Data are presented as Mean ± SD, or frequency (percentage).

Table 2.
The laboratory finding of the tumor markers and ultrasound examination of the studied cases after mesenchymal stem cells therapy.

		All (n = 100)
ALDH		183.4 ± 31.5
AFP		3.16 ± 1.6
CEA		2.27 ± 1.1
B. HCG		0.95 ± .2
Scrotal ultrasound	No focal	100%

Data are presented as Mean ± SD, or frequency (percentage). LDH: Lactate Dehydrogenase; AFP: Alpha-Fetoprotein; CEA: Carcinoembryonic Antigen; HCG: Human Chorionic Gonadotropin.

Discussion

Inability to achieve pregnancy despite having regular sexual intercourse for 12 months is defined as "*infertility*" (10). This pathology affects approximately 8-12% of married couples of reproductive ages worldwide. The most severe form of male infertility is NOA, which is the absence of spermatozoa in the ejaculate due to impaired spermatogenesis. Therapies for male infertility fall into several categories, including optimization of sperm production, relief of obstruction, and surgical sperm retrieval (11).

MSCs, a class of highly heterogeneous cells that can be isolated from bone marrow, adipose tissue, the umbilical cord and the placenta, were primarily discovered in 1974 by *Friedenstein* (5).

Clinically, MSCs have been applied in many refractory diseases, such as cerebral palsy (12), spinal cord injury (13), systemic lupus erythematosus (14), reproductive diseases including azoospermia (15). However, MSCs have been shown to aggregate easily, potentially forming the core of microthrombi which contributes to vascular complications. Moreover, due to their proliferative capacity, MSCs carry a theoretical risk of tumorigenicity and may elicit acute or chronic immunogenic responses under certain conditions. Many studies, most of which have enrolled small sample sizes, have investigated the safety of *mesenchymal stromal/stem cell* (MSC) transplantation. Although a limited number of human studies such as the Phase I clinical trial by *Khadra et al.* (2022) have explored short-term safety outcomes, including tumor marker monitoring, no comprehensive reviews have been conducted to evaluate the adverse effects or the long-term tumorigenic potential of MSC transplantation (16). Therefore, the objective of the study was to investigate the safety and potential carcinogenic effects of autologous hBM-MSCs implanted into the testes of patients with NOA.

According to our research, there is no significant malignancy detected post- MSCs therapy as indicated by the normal level of LDH, AFP, CEA, and β-HCG.

Additionally, scrotal ultrasound showed no focal findings in all cases, indicating the absence of structural abnormalities, such as tumors or lesions in the testicles.

Several studies have explored the use of different types of stem cells, such as MSCs (17) and *spermatogonial stem cells* (SSCs), in the treatment of azoospermia (15, 17). MSCs, known for their differentiation capabilities, have shown potential in promoting the regeneration of damaged testicular tissue (18). However, despite the excitement surrounding stem cell therapy, there are significant challenges that must be addressed. One major hurdle is the precise regulation of stem cell differentiation into functional sperm cells. Ensuring that the differentiated cells contribute to sperm production without forming tumors or causing unwanted side effects is a complex task (19).

The therapeutic effect and frequency of complications after multipotent cell therapy remain questionable (20). The therapeutic effects are generally based on the anti-inflammatory effect caused by prolonged paracrine activity of injected cells. However, multiple complications can be explained by the mesenchymal phenotype causing their migration and adhesion (21).

The current findings are supported by a Phase I clinical trial conducted by *Zhankina et al.* (22), which evaluated the therapeutic effect of autologous *bone marrow-derived mesenchymal stromal/stem cell* (BM-MSC) transplantation into the testes of patients with NOA. In this study, 80 patients underwent intratesticular MSC injection during micro-TESE and were followed for six months. The investigators reported no adverse events related to tumor development or abnormal tissue growth. Importantly, all patients maintained normal tumor marker profiles including LDH, AFP, β-HCG, and CEA throughout the follow-up period. These findings suggest that autologous MSC therapy may be safe in the short term, particularly due to the immunocompatibility of using the patient's own cells. However, the study's limited sample size and relatively short follow-up highlight the need for longer-term investigations to fully assess potential tumorigenic risks (22).

In addition, The *Canadian Critical Care Trials Group* has recently published a meta-analysis of randomized, non-randomized, controlled and uncontrolled, phase I and phase II clinical trials (23); no association between autologous or allogeneic MSCs administration and tumor formation was reported in the 36 studies reviewed by them. Nonetheless, longer follow-up is required to draw a final conclusion regarding human MSCs' tumorigenic potential.

The malignancy of MSCs has been reported only by *Ning* (24) who investigated MSCs transplantation in hematologic malignancy patients and found that the relapse rate was significantly higher than the control group treated with hematopoietic stem cells.
Further, some animal and in-vitro studies showed that MSCs administration may promote growth of different tumors through a variety of mechanisms, including expression of proangiogenic factors and immunosuppression (25).
Our study was limited by the retrospective design, which allowed for the possibility of missing data. Additionally, it was a single-center study with an insufficient sample. Also, additional molecular studies on the molecular and cellular mechanism of MCSs in the testes would provide deeper insights into their potential therapeutic benefits and risks.

Conclusions

This retrospective study found no evidence of malignancy or structural abnormalities two years after intratesticular injection of autologous bone marrow-derived MSCs in NOA patients, based on tumor marker profiles and scrotal ultrasound. These findings support the medium-term safety of this approach and offer encouraging insights for further research into stem cell-based therapies for male infertility. However, larger prospective studies with extended follow-up are needed to confirm long-term safety and therapeutic efficacy.

Declarations

Ethical approval and consent for participate: *All procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the appropriate local institutional ethical committee.*
Ethical approval numbe : RC 23/3/2011

Consent for publication: *All cases were volunteers and informed by explaining complete details of the procedure. Consent was obtained from each patient.*

Availability of data and material: *Sequence data that support the findings of this study are available upon request.*

Competing interests: *The authors have no competing interests to declare that are relevant to the content of this article.*

Funding: *This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.*

Authors' contributions: *All authors contributed to data acquisition, study conception and design; Material preparation, data collection and analysis were performed by Mohamed Abdelrahman Alhefnawy, Mohamed Aboulfotouh Elgharably, Gamal Zakaria Elmorsy Elkhatib, Hesham Atia Soliman El-amrosy, Taymour Mohamed Khalifa, Hany Sabry Ahmed Ibrahim and Helmy Ahmed Eldib; Helmy Ahmed Eldib wrote the first draft of the manuscript, and all authors commented on previous versions of the manuscript.All authors read and approved the final manuscript.*

Acknowledgments: *None.*

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