Mesenchymal Stem Cells Restore Fertility in Induced Azoospermic Rats Following Chemotherapy Administration

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Abstract: Impairment of spermatogenesis has been confirmed before treatment in patients with various malignancies. Also, testicular germinal epithelial damage resulting in azoospermia is a well-known consequence of certain chemotherapeutic agents. The chance of recovery of spermatogenesis following cytotoxic insult and also the extent and speed of recovery, are related to the agent used and the dose received. Transplantation of mesenchymal stem cells (MSCs) which are able to self-renew to maintain the stem cell population and activate the resident testicular stem cells, producing large numbers of differentiating cells of the spermatogenic line, eventually leading to mature spermatozoa that will transmit the genome to the next generation. This study was designed to assess the ability of mesenchymal stem cells (MSCs) to restore fertility in induced azoospermic rats following Busulfan administration. Forty male rats were divided randomly into four groups (10 rats each). Group 1, representing control rats received phosphate buffer saline, group 2 including azoospermia induced rats by Busulfan, group 3 including azoospermic rats that undergo transplantation MSCs into the right testis after induction of azoospermia and group 4 for bone marrow MSCs donation. Testicular size androgen hormonal profile and histological assessment of testicular tissues were evaluated pre- and post- mesenchymal stem cell transplantation. Results revealed that MSCs after 12 weeks of transplantation to induced azoospermic rats; are able to testicular size androgen hormonal profile levels and resuming spermatogenesis as verified by histological assessment of testicular tissues as compared to control groups and evaluated pre- and post-mesenchymal stem cell transplantation. In conclusion, MSCs have potentials for trans-differentiation into germ cells in vivo in testicular microenvironment. also, it able to restore testicular functions as well as increase testicular size.

Key words: Busulfan • Mesenchymal Stem Cells • Spermatogenesis • Azoospermia

INTRODUCTION

Impairment of spermatogenesis due to the failure in germ cell proliferation and differentiation is considering one of the major factors of male infertility. In normal healthy couples pregnancy rate by intercourse is reached 20-25% within one month, 75% within six months and 90% within a year. However, male factor is involved in 50% of infertile relationships. In particular, men with nonobstructive azoospermia have little or no sperm in their semen and cannot have their own biological children. Patients with nonobstructive azoospermia are typically characterized as having small-volume testes, decreased testosterone levels, lacking of sperm and elevated FSH [1, 2].

Germinall epithelial damage is a recognized consequence of certain chemotherapeutic agents and radiotherapy which represented by absence of spermatogenesis in the seminiferous tubules this was observed in 27 of 30 men treated for lymphoma with nitrogen mustard [3] and similar reports of testicular toxicity due to alkylating agents, such as Busulfan, chlorambucil and cyclophosphamide [4].
Busulfan (1, 4-butanol dimethanesulphonate) is often used as a conditioning regimen prior to progenitor cell transplantation for treatment of chronic myelogenous leukemia and can induce prolonged azoospermia [5]. The cytotoxic effects of Busulfan takes place via formation of DNA–DNA cross-links, DNA–protein cross-links and single strand breaks. It also, exerts its toxic effects on cells that are at the G1 phase at the moment of treatment. These cells are killed in the following mitosis, while those that are in S or G2 phase are killed in the subsequent mitosis [6]. In the testis, Busulfan preferentially kills spermatogonia of several species, leading to male infertility [7]. Even when administrated to pregnant animals, Busulfan produces germ-cell-free gonads in the offspring [8, 9].

Mesenchymal stem cells (MSCs) are widespread in adult organisms and may be involved in tissue maintenance and repair, as well as in the regulation of homeostasis, immunological responses and function restoration [10, 11]. Bone marrow derived mesenchymal stem cells (BM-MSCs) are different from other somatic stem cells in that they differentiate not only into the same mesodermal lineage but also into other lineages with different embryonic origins such as bone marrow, muscle, liver, lung and skin [12-14]. Various authors reported generation of germ cells from uncommon sources, such as mesenchymal stem cells has been of particular interest in recent years due to their significant clinical applications. Recent studies have shown the ability of both embryonic [15, 16] and adult stem cells [17, 18] to differentiate into primordial germ cells and further mature gametes. By day 6 after birth, primordial germ cells (PGCs), the origin of male germ cells that arise from proximal epiblast, migrate to the basement membrane of the seminiferous tubules and become spermatogonial stem cells (SSCs) [19]. SSCs are responsible for maintaining the spermatogenesis process throughout the life time [4, 20, 21] A key step in investigation of male infertility is the appropriate classification of impaired spermatogenesis [20, 22]. Mesenchymal stem cells possess the capacity to trans-differentiate into epithelial cells and lineages derived from the neuro- ectoderm and in addition, these cells can migrate to the sites of injury, inflammation and to tumors. These properties of mesenchymal stem cells make them promising candidates for use in regenerative medicine [13]. A recent study has demonstrated that bone marrow stem cells are able to differentiate into primordial germ cells and spermatogonia both in vitro and in vivo [15]. In addition, another study has demonstrated that adult stem cells derived from bone marrow stroma can differentiate into Leydig cells in rat testes [23].

So, this study aimed to investigate the regenerative potency of bone marrow derived-mesenchymal stem cells (BM-MSCs), implanted into the induced azoospermic rats testis. Using various predictive parameters such as histological examination of spermatogenesis in testicular tissues and physiological parameters related to male reproduction (testis weights and volumes, hormonal (testosterone, L.H and FSH) for monitoring the degree of spermatogenic recovery.

**MATERIALS AND METHODS**

**Experimental Design:** 40 Male Sprague-Dawley rats 8 weeks old, weighing 150–180 g, were obtained from Holding Company for Biological Products & Vaccines (Vacsera), Helwan, Egypt and maintained in the Experimental Animal Unit, Faculty of science, Al Azhar University. The recipient animals received a standard laboratory chow and water ad libitum. Rats were divided randomly into four groups of 10 males each: group1 involved control rats received phosphate buffer saline, group 2 included rats with experimental azoospermia induced by administration of Busulfan, group 3 included azoospermic rats that underwent transplantation MSCs into the testis after induction of azoospermia and group 4 for bone marrow MSCs donation. Animal carcasses were disposed of by incineration in accordance with guidelines stated by Institutional Animal Care and Use Committee (IACUC) guidebook found at NIH website http://www.grants.nih.gov/grants/olaw/ guidebook.pdf

**Recipient Animals:** Recipient animals were induced with a system of divided injections was used in which 10 then 15 mg/kg Busulfan (GlaxoSmithKline Inc., Mississauga, Ontario, Canada) was administered by intraperitoneal injection with a 14 day interval [24]. MSCs transplantation was performed 6 weeks after the initial Busulfan injection to induce testicular degeneration (experimental azoospermic animal model). The effectiveness of this process was determined first by measuring testis size, weight and histological examination. All experiments in this study take place in the Center of Genetic Engineering, Faculty of science, Al Azhar University- Cairo. Egypt.
Isolation and Expansion of Bone Marrow Derived Mesenchymal Stem Cells (MSCs): Briefly, MSCs were isolated according to a protocol modified from Short and Wagey [25] by flushing femurs and tibias of 4–6 week old male rats. The mononuclear cells were isolated from bone marrow cells suspension using Ficoll (Ficollhypaque, Sigma–Aldrich, St Louis, MO). Then cultured on DMEM-LG supplemented by 15% fetal bovine serum (Gibco® Sera), penicillin/streptomycin (50 U/ml and 50 mg/ml, respectively; Sigma-Aldrich, St Louis, MO) supplemented with heparin at a final concentration of 5 U/ml at37°C and 5% CO2. MSCs expanded normally with a spindle appearance and were readily grown in vitro by successive cycles of trypsinization, seeding and culture.

Labeling of Bone Marrow-derived Mesenchymal Stem Cells: MSCs cells were labeled with fluorescent cell tracer PKH26 (Sigma-Aldrich Co, St. Louis, MO, USA) this lipophilic red fluorescent dye stably integrates into the cell membrane. PKH26 is a red fluorochrome. It has excitation (551 nm) and emission (567 nm) characteristics compatible with rhodamine or phycoerythrin detection systems. In the current work, MSCs cells were labeled with PKH26 according to the manufacturer’s recommendations (Sigma).

Surgical Process and MSCs Transplantation: MSCs cells concentration for transplantation was 1X10^8 cells/ml suspension was mixed with sterile 0.04% trypan blue stain (Invitrogen Corp) and transplanted into the recipient rat through the efferent ducts into the seminiferous tubules as described before [18]. A total of ten animals received the 100 µL cell suspension in one testis (right) while the other (left) was used as internal control, receiving 100 µL of PBS.

Analysis of Recipient Rats
Testis Weight and Volume: Testis weights were measured at the time of euthanasia without removing the tunica, the volume of testis was estimated by Cavalieri’s principle via Vernier caliper.

Histological Assessment of Spermatogenesis: The animals were sacrificed and their testes were removed and fixed in 10% neutral buffer formalin. The fixed samples were then embedded in paraffin and sectioned (5µm thick) on gelatin precoated slides. They were further deparaffinized, stained with Hematoxylin–Eosin and observed under a light microscope (Carl Zeiss).

Hormonal Assessment: To determine serum testosterone, L.H and FSH levels, 1–2 ml of blood was collected from the jugular veins of rats before euthanasia, centrifuged to isolate serum and stored at – 80°C until analyses. Testosterone, L.H and FSH levels were measured using the ELISA kit (BioCheck, Inc., USA catalog No: BC-1115, BC-1111 BC-1113 respectively) according to the manufacturer’s instructions.

RESULTS

Culture of BM-MSC: MSCs attached to the culture flasks sparsely and displayed a fibroblast-like, spindle-shaped morphology during the initial days of incubation. Following 3-4 days of incubation, proliferation started and the cells gradually grew into small colonies. During culture, adjacent colonies interconnected with each other and a monolayer confluence was obtained after 12–15 days of incubation. In later passages, MSCs exhibited large, flattened fibroblast-like morphology (Figure 1a-f) and did not change throughout 9 passages. The effect of seeding density was reflected also in cell morphology changing from thin spindle-like shape in low densities into flattened appearance in dense cultures. The colonial growth pattern was also most prominent in low density cultures (Fig. 1c&f).The viability of cells was higher than 95%, determined by Trypan blue staining of cells before transplantation.

Histological Assessment of Spermatogenesis: Formalin fixed and paraffin-embedded testis tissue sections were stained with hematoxylin-eosin. In the control group at time of stem cells transplantation into azoospermic rats, most of the seminiferous tubules showed a thick wall with a very limited lumen (Figure 2 A&B). The wall consists of several layers of seminiferous epithelial cells, with the spermagonia in the outer layer, the spermatocytes in the middle and the spermatozoa and spermatids protruding toward the lumen, which represent ‘fully spermatogenic’ seminiferous tubule.

After induction of azoospermia in rats using 10 then 15 mg/kg Busulfan administered by intraperitoneal injection the testes of the animals were thoroughly analyzed for any spontaneous recovery of spermatogenesis and no sign was observed for reinitiated spermatogenesis. The scanning of cross sections of these tubules showed reduced diameters with small lumens and contained few spermatogonia. Nuclei of Sertoli cells had become smaller
Fig. 1: Morphological and phenotypic characteristics of rBM-MSCs. During the onset of culture (A & B) P0-3th day, the isolated cells from rat bone marrow, some of the cells are still spherical in shape, while others begin in the formation of fibroblast shape. (C&D) Prominent alterations in morphology were documented after 1 week of culture after the next days and passages, most of these MSCs exhibited large, flattened or fibroblast-like morphology. (E&F) A colonial growth pattern was prominent at higher density after 3 weeks. Light inverted microscope, Scale bars: (A-B=500 µm), (C-F=100 µm)

Fig. 2: Cross section of testis
Photomicrographs of cross-sections from Sprague-Dawley male rat’s testes (A): Testis of control animals 8 weeks showing a typical fully normal appearing spermatogenesis organization in seminiferous tubules. (B): Higher magnification of A, showing a Seminiferous Tubules (ST) with complete spermatogenesis process. Healthy sperm (S), tubular lumen (L), interstitial tissue containing Leydig cells (LC), spermatogonial stem cells (SSC), primary spermatocyte (PS), Secondary Spermatocytes (SS), round spermatides (RS), Sertoli cells (SC) and blood vessel (BV). (C): showing germ cell loss is seen in significant numbers of tubule sections and damage is more evident. (D): Higher magnification of C, showing a seminiferous tubules showing a typical non-spermatogenesis, the population of spermatogonia has decreased markedly and many tubules contained only Sertoli cells (arrows).
Scale bars: A: 100 µm, B&D: 40 µm and C: 250 µm
Fig. 3: Restoration of spermatogenesis in infertile induced azoospermic rat by the transplantation of BM-MSCc cells into testes. (A): Tubular cross section of a testes from a recipient rat 3 months after rat donor MSCs transplantation. Note that most of the tubules show spermatogenesis and more advanced germ cells were found. (B): The same section as in (A) at a higher magnification. (C & D): PKH26-labelled donor germ cells in the seminiferous tubules of recipient rat after 3 months of transplantation. Mesenchymal stem cells (MSCs) labeled with the PKH26 showed strong red auto-fluorescence after transplantation into rats, confirming that these cells were seeded into the seminiferous tubules. (E) left testes (injected with PBS and remains as internal control), (F): The same section as in (E) at a higher magnification almost all seminiferous tubules were degenerated and neither recovery is noticed or any fluorescent cells record (Data not shown).

Scale bars: B, D & F = 40 µm. A = 200 µm, C & E bars = 100 µm.

Table 1: Changes in testis weight, length and width of adult male rats after and before MSCs transplantation.

<table>
<thead>
<tr>
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<th>Control group</th>
<th>Busulfan induced group</th>
<th>Stem cells group (MSCs)</th>
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<tr>
<td>Right testis</td>
<td></td>
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<tr>
<td>Weight</td>
<td>1.07±0.05</td>
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<tr>
<td>Left testis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight</td>
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<td>0.55±0.05</td>
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Values are represented as Mean± SE, NS = non-significant, * = significant (P < 0.05) and ** = highly significant (P < 0.01)

and the cells were irregularly located, large amounts of interstitial fluid were observed between the tubules as an eosin-stained homogenous substance. Atrophy and complete spermatocytic arrest were developed and azoospermic model become ready for MSCs transplantation (Figure.2 C&D).

Histological analysis of the right testes 12 weeks after MSCs transplantation showed that spermatogenesis was re-established. Transplanted testes tubules’ cross-sections showing degeneration in some and restored spermatogenesis in others, these latter show all the stages of the spermatogenic cycle (Figure.3 A&B). Also fluorescence examination it was observed that transplanted rats presented differentiated germ cells stained with fluorescent cell tracer PKH26, 12 weeks after cell transplantation. And this is a good evidence revealed that these cells are perfectly inserted on the seminiferous epithelium (Fig. 3 C&D). On the other hand, in left testes (injected with PBS) almost all seminiferous tubules were degenerated and neither recovery is noticed nor any fluorescent cells record (Figure. 3 E&F).

Testicular Weight, Volume (Length & Width) and Spermatogenesis: Testis weights declined after Busulfan treatment to 50% of the pretreatment level by the time of fertility loss (6 weeks) in both groups (2 & 3), which were treated Busulfan to induce infertility in which then we use of group 3 (recipient rats) for the MSCs transplantation (Table 1). Thereafter, the weights gradually increased in the right recipient testes that recovered after 12 weeks of MSCs transplantation. The difference in dimensions (length & width) also noticed after Busulfan treatment in comparison with control group and recipient testes. It was observed that the average volume of the right testicles with MSCs was higher than the ones with no transplantation and atrophy was not seen in testicles with MSCs transplantation. The volume increase of Busulfan-treated testes was only observed significantly in the test subjects with MSCs transplantation; a significant increase of size in other interstitial tissues was not noticed.
Hormonal Assessment of Spermatogenesis: Serum hormone assays show that testosterone level has an increase as compared to the control group (Fig. 4a), despite the increase in serum testosterone level in Busulfan group there was any significant difference among other groups, regeneration of spermatogenesis was not observed. Also hormonal assessment demonstrated that FSH shows significant differences in Busulfan group than those of the other two groups (P < 0.05). On other hand LH increased in the serum level compared to the control group, but no statistically significant difference was detected between the LH in Busulfan, MSCs and the control group (Fig. 4b). These observations indicated that the effects of LH and FSH on spermatogenesis were synergistic.

DISCUSSION

The present study was carried out to assess the regenerative capacity of rat bone marrow derived-mesenchymal stem cells (BM-MSCs) transplantation into the induced azoospermic rat testes after 12 weeks of follow up stem cells were able to recover testicular functions and size. Testis represents a unique microenvironment for donor stem cell migration, proliferation, differentiation and apoptosis. The testis is protected from immunological influences by the blood-testis barrier allowing the recipient to host donor cells without rejection [12].

Histological examination in the our study showed that at 6 weeks after Busulfan injection, more than 95% of the seminiferous tubules had become non-spermatogenic, with the depletion of about 95% of the haploid cells (the spermatids and spermatozoa) in the animals treated with 10 then 15 mg/kg Busulfan. These results run with that published by Jackson and Ogawa [24, 32] who revealed that the system of divided injections is the accurate way for azoospermic model design to study cell transplantation into recipient animal because Busulfan of 25 mg/kg is a lethal dose in rats due to their myelotoxicity [26].

The donor cells used in this study were fractionated bone marrow cells containing mesenchymal stem cells; previous reports demonstrated that bone marrow-derived mesenchymal stem cells are capable of differentiating into germ cells and Leydig cells in the testis [15, 16], Yazawa and Nayernia, [17, 23]. On the contrary, Lue, [12] and Horn, [27] were used donor cells asunfractionated bone marrow cells containing hematopoietic stem cells, endothelial stem/progenitor cells, mesenchymal stem cells and multipotent adult progenitor cells. Stem cell transplantation is usually performed 4–6 weeks after treatment. The donor cells usually require 4 weeks after transplantation to attach to the basal lamina and proliferate [23] and an additional 4-8 weeks to produce complete seminiferous epithelium with spermatids and spermatozoa as described by Sabbaghi [27].

It was unexpected to us that there was a high proliferative and strong ability to recovery of induced azoospermia during fertility restoration and this finding become clear in histological examination and fluorescence activity of PKH-26 as in also very obvious changes in the size and weight of the testis is unequivocal evidence of the regeneration process or not which shows significant differences in Busulfan treated testes in both right and left testes (P<0.01) suggesting that Busulfan causes complete
atrophy in recipient testes and MSCs transplantation attempts to recover seminiferous tubules. Such finding coincides with that obtained by Cakici, [2] whom demonstrated that seminiferous tubules recovery after adipose tissue derived mesenchymal cells transplantation not completely recovered in spite of breeding production. The cause of this discrepancy is unclear, but the difference in number of transplanted cell strains or donor and recipient mice between the two studies could have contributed to it. On other hand good evidence revealed our study that the remaining un-transplanted left testes (Busulfan treated) still azoospermic among recovery period performed in right transplanted testes and this observations becomes on the contrary with finding of Lassalle, [11].

Although an increasing amount of evidence supports our finding is the reduced serum LH level leads to suppression of testosterone production in the testis. It is speculated therefore that lowered testosterone could be relevant to the promoted regeneration of spermatogenesis [26]. A series of investigations suggested that an excess of testosterone inhibited the proliferation of spermatogonia [28, 29]. When spermatogenesis is impaired by irradiation or chemical agents, the serum LH level is usually elevated [14]. The high LH level increases vascular permeability, which increases ITF(intra testicular fluid) and induce edematous change in the testis [22]. Regular doses of testosterone, which are now commonly used in clinics, suppress spermatogenesis by decreasing the secretion of gonadotropins from the pituitary gland. Therefore, testosterone treatment in normal males, as well as GnRH analogs, regularly produces testicular atrophy and azoospermia [30]. This could be attributed to describe our results in Busulfan induced group that shows elevated testosterone level in attempts to recover depletion due to Busulfan induction. However, this exogenous testosterone, unlike GnRH analogs, does not affect the weights of sex accessory organs because it is supplementary to reduced testosterone production in the testis. [42, 43]. Follicle stimulating hormone (FSH) and testosterone are the main hormonal regulators of spermatogenesis. Both hormones are necessary for the initiation of spermatogenesis [31]. The synergistic effects of FSH and estradiol on the regeneration of spermatogenesis have been shown [10] together with previous observations and our results suggest that the significant changes in FSH during hormonal assessment in agreement with testosterone elevation explains closed correlation between FSH and testosterone in their role of spermatogenic recovery after and before MSCs transplantation. Finally, the present finding may have a major impact in understanding reproductive physiology and recovery from testicular pathology and also may introduce a novel future therapy for azoospermic patients.

REFERENCES


