

Original Paper

Effects of Sertoli Cell Transplantation on Spermatogenesis in Azoospermic Mice

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Key Words

Sertoli cells • Spermatogenesis • Busulfan • Genes • Reproductive

Abstract

Background/Aim: The aim of this study was to evaluate the potential and significant applications of Sertoli cells (SCs) transplantation, and to explore the effect of transplantation on spermatogenesis process, in azospermic mice. **Methods:** In this study, we utilized 18 adult mice (28–30 g), divided into four experimental groups: (1) control, (2) vehicle (DMSO 2%) (10 µl) (3) busulfan and (4) busulfan+ SCs (1×10⁴ cells/µL). SCs were isolated from the testis of 4-week-old mouse and after using anesthetics, 10 µl of SCs suspension (1×10⁴ cells/µL) was injected over 3–5 min, into each testis and subsequently, sperm samples were collected from the tail of the epididymis. Afterward, the animals were euthanized and testis samples were taken for histopathology experiments, and RNA extraction, in order to examine the expression of c-kit, STRA8 and PCNA genes. **Results:** Our data showed that SCs transplantation could notably increase the total sperm count and the number of testicular cells, such as spermatogonia, primary spermatocyte, round spermatid, SCs and Leydig cells, compared to the control, DMSO and busulfan groups. Furthermore, the result showed that the expression of c-kit and STRA8 were significantly decreased in busulfan and busulfan/SCs groups, at 8 weeks after the last injection (p<0.001), but no significant decrease was found for PCNA, compared to the control and DMSO groups (P<0.05). **Conclusion:** These findings suggest that SCs transplantation may be beneficial as a practical approach for therapeutic strategies in reproductive and regenerative medicine. We further highlighted the essential applications that might provide a mechanism for correcting fertility in males, suffering from cell deformity.

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Introduction

Spermatogenesis or seminiferous epithelial cycle is a complex and extraordinarily productive process, including the mitosis, meiosis and spermiogenesis of spermatogonia, spermatocytes and spermatids, respectively. In addition, it affects the pathogenesis mechanisms of the cellular process, including the program of various developmental stages of the germ cells. In parallel and at the beginning, by meiotic division, it differentiates to spermatocytes cells and afterwards through meiosis, these cells transform to round spermatids and eventually spermatozoa will be released into the lumen, via elongated spermatids [1-7].

All these spermatogenesis processes commonly occur in the enclosed microenvironment pool in germ cell development that are deployed in the seminiferous tubule of the testis [8-11]

The whole process of spermatogenesis relies on functional interplays, among germ cells and somatic, supporting cells (Sertoli cells (SCs), involving a complex classification of hormones, cytokines and vitamins [12, 13]. SCs or “mother cells” or “nurse cells” play a principal role in regulating and controlling spermatogenesis by physiological and structural contacts, immunological and nutritional support, notably it can be baldly transformed into Leydig cells, and also changed to the neural stem cells of morphologic, phenotypic and functional type [14-17]. In addition, these cells have the ability for self-renewal, proliferation and spermatogonial stem cell differentiation, due to having a crucial component of different factors, including glial cell-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), kit ligand (KITL), activin A and bone morphogenic protein 4 (BMP4). Furthermore, SC function is strongly controlled by FSH and testosterone signaling. Moreover, SCs are engineered to produce specific proteins that reconstitute the metabolic function of the cell. Thus, SCs can be utilized in the modeling of diseases and regenerative medicine [6, 17-22].

Multiple reports of *in vivo* studies regarding gene therapy, using SC, independently suggested that this cell could invert the infertile phenotype in “Steel” mice [23-25]. In addition, another study by Zhang *et al.* (2007) revealed that donor SC from immature rats, colonized within the seminiferous tubules, but they have not found the effects of SC on spermatogonial differentiation [26]; however, the successes in these models may propose that *in vivo* SC gene remedies could be expanded to treat infertility of men with somatic defect [27].

In this report, in order to elucidate the potency of SC and to explore its potential role, we highlighted the potential and significant applications of SC transplantation in azospermic mice, and examined the effect of transplantation on spermatogenesis process.

Materials and Methods

Animal models

In this study, 40 adult mice 8 weeks of age, weighing 28–30 g were used. The animals were purchased from the laboratory animal center of Pasteur Institute, Tehran, Iran. The Ethics Committee of the University approved the animal experiment (IR. SBMU. SM.REC.1396.184). The mice were divided into four experimental groups: (1) control, (2) vehicle (DMSO 2%) (10 µl) (3) busulfan and (4) busulfan+ SCs (1×10^4 cells/µL). Each group harbored ten mice, housed under standard conditions (room temperature 22°C–24°C and the 12:12 hour light-dark schedule). The mice had free access to water and food.

Busulfan treatment

Mice were induced with Azospermia and oligospermia, by injection of a single dose of 40 mg/kg busulfan (Sigma-Aldrich Product Number B2635), intraperitoneally. All mice were kept for 8 weeks after the busulfan injection, and then the sperm was analyzed.

Isolation and culture of Sertoli cells

The SCs were isolated from testis of 4-week-old male mice. Then, enzymatic digestion and decapsulation was performed, using 0.25% trypsin (Sigma) and 0.1% collagenase (Sigma, type V) at 37°C. Then, the SCs were transferred into cell culture flasks and kept in the cell culture incubator. After 24 h, the cell culture flasks were washed to eliminate debris. After 48 h, the hypotonic cells were treated for 2.5 minutes with 20 mM Tris-HCl (pH 7.4), to lyse any residual germ cells and to get SCs with the purity of >95%.

Labeling of SCs with Hoechst

We applied Hoechst solutions for labeling SCs. First, 5 μ l of Hoechst 33258 (Sigma) solutions added to 1 ml of serum-free cell suspension (1×10^6 cells/mL). Then, it was incubated for 20 minutes at 37°C to prepare uniform labeling. Following the centrifuge and re-suspension of cells in a warm medium, labeled SCs suspension was ready for transplantation. The cells from the first labeled passage were collected, when they reached to 90% confluency, and then washed with PBS (Fig. 1).

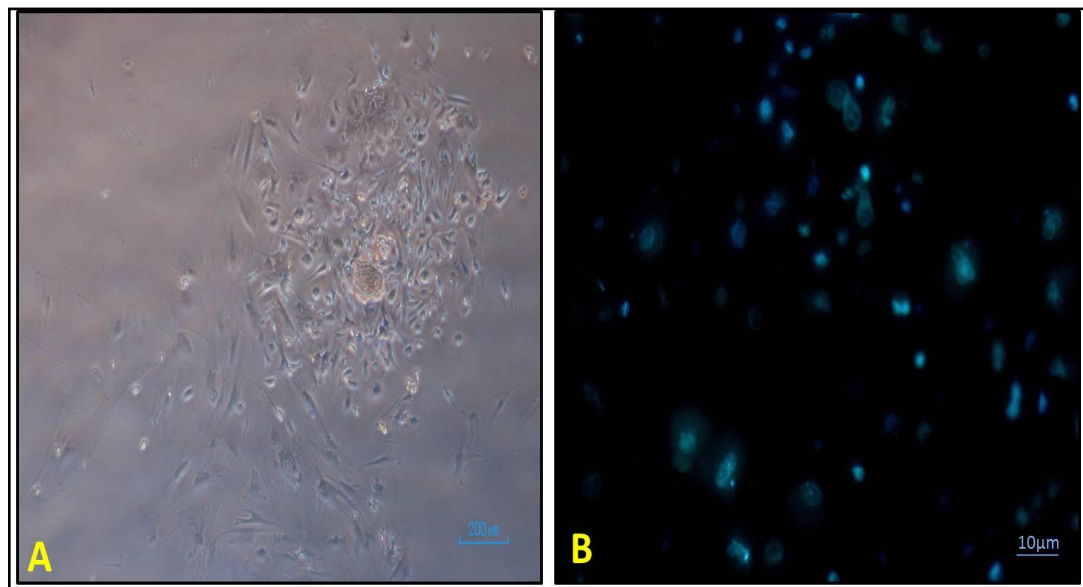


Fig. 1. (A) Sertoli Cells after 7 days culture (B) Fluorescent labeling with Hoechst for Sertoli cells culture.

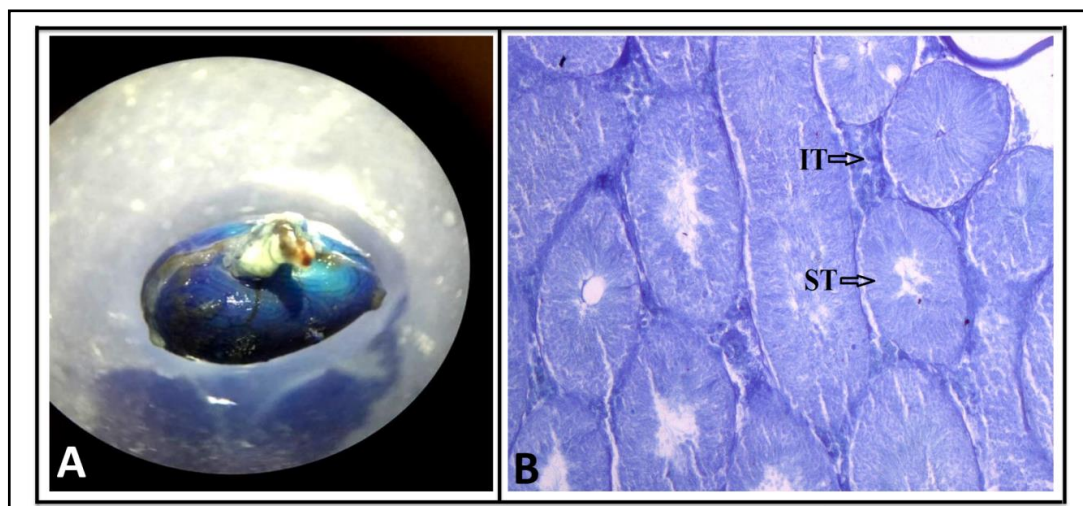


Fig. 2. (A) Toluidine blue dye was injected in testis as control. (B) Blue staining indicates seminiferous tubule (ST) and interstitial tissue (IT).

Transplantation

Before transplantation of SC, in order to examine the trace of injection, toluidine blue dye was injected in testis, as a control. After reassurance of suitable injection site, mice in the experimental group were anesthetized by ketamine (100 mg/kg) and xylazine (5 mg/kg). Then, 10 µl of SCs suspension (1×10^4 cells/µL) was injected over 3–5 min, into each testis (Fig. 2).

Sperm collection

Sperm samples were collected from the tail of the epididymis and then transferred to the 1 ml of Ham's F-10 media (Sigma-Aldrich Product Number N6635). After incubation at 37 °C for 20 minutes, 10 µl of the sample was placed on a slide and sperm motility was observed. The sperm count was measured by counting chamber. For examination of morphology, the sperm smear was prepared for analysis, placed on a slide, air dried at room temperature, and fixed in methyl alcohol. Then, the sample was stained with Diff-Quik.

Stereological study

On days of sampling, animals were euthanized with ether and their testes were collected and fixed in paraformaldehyde 4%. After fixation, samples were sectioned by rotary microtome into 5 µm thickness slices, stained with hematoxylin and eosin (H &E), and examined by light microscopy.

Estimating the volume of testis

Total volumes of the testis and interstitial tissue were measured by the Cavalieri method. The Cavalieri method [28] was used as an estimator of the testis volume. Thus, 8–10 sections were selected, using a systematic uniform random sampling. The image of each section was evaluated, using the stereological software and a video-microscopy system, made up of a microscope (Nikon, E-200, Japan), linked to a video camera. The volume of the testis was estimated by the following formula [29, 30]:

$$V_{\text{total}} = \sum P \times t \times \frac{a}{p}$$

In this formula $\sum P$ is the total points, hitting the testis sections, a/p is the area associated with each point, and t is the distance between the sampled sections.

Estimating the length of seminiferous tubules

To estimate the length of seminiferous tubules, six random fields from sections were selected and the number of the selected seminiferous tubule profiles was counted, using an unbiased counting frame [31–33]; thus, an average number of seminiferous tubules per testis, was estimated. The length density of seminiferous tubules was also estimated, using the following formula [29, 30]:

$$L_v = \frac{2 \times \sum Q}{\sum P \times \frac{a}{f}}$$

In this formula $\sum Q$ is the total number of the seminiferous tubules, a/f is the area per frame and $\sum P$ is the total points superimposed on the testis tissue. The total length of the seminiferous tubules was estimated by multiplying the length density (L_v) by the total volume of the testis.

Estimating the number of spermatogonia, spermatocyte, spermatid, SCs and Leydig cells

The number of spermatogonia, primary spermatocyte, spermatid, SCs and Leydig cells were determined, using the optical dissector method and an unbiased counting frame. The position of the microscopic fields was selected by systematic, uniform random sampling, with moving the stage in equal distances in x- and y-directions. The focal plane was moved downwards in z-direction. Then, a microcator was attached to the stage of the microscope (Heidenhain, Germany) to measure the z-axis traveling

Table 1. Primers design

Genes	Primer sequences	Product size	TM (°C)
c-Kit	F: GCATCACCATCAAAAACGTG	332bp	58
STAR	F: GCCAGAATGTATTCCGAGAA	121bp	57
PCNA	F: GATGTGGAGCAACTTGGAAT	160bp	53
GAPDH	F: ATCACTGCCACTCAGAAGACTG	293 bp	60

(depth). Using the following equation, the number density (Nv) of different types of cells was estimated, using the following formula [29, 30]:

$$N_v = \left[\frac{\sum Q^-}{\sum P \times \frac{a}{f} \times h} \times \frac{t}{BA} \right]$$

In this formula $\sum Q$ is the total number of the counted cells, h is the tissue thickness considered for counting, a/f is the area per frame and $\sum P$ is the total number of the counting frames in all fields. H is the height of the dissector, t is the real section thickness measured, and using the microcator, when the Q^- is counted and BA is the tissue section thickness. The result of the equation was then multiplied by the total volume of the testis, to obtain the total number of cells.

$$N_{(total)} = N_v \times V_{(final)}$$

Tunnel assay

DNA fragmentation refers to apoptosis markers. Terminal deoxynucleotidyl transferase dUTP nick and labeling (Tunnel) is one method to detect DNA fragmentations. After treatment, the tissues were fixed and embedded in paraffin, then sectioned on glass slides, and stained according to the Tunnel protocols. Finally, Tunnel-positive cells were quantified in 10 sections per testis.

RNA extraction

The High Pure RNA Isolation kit was considered to isolate the total RNA from the mice testes, following the manufacturer's instructions (Roche, Basel, Switzerland). Subsequently, the collected RNA samples were treated with DNase I enzyme. Afterward, the Nanodrop ND-1000 spectrophotometer (Thermo-Scientific, USA), with an absorbance of 260 nm and 280 nm, and agarose gel electrophoresis (2%) were used to evaluate the purity of total harvested RNA.

Primer design

All primers were designed, according to the Primer3Plus software, considering the exon-exon junction, to discriminate between cDNA and genomic DNA, when possible. Formerly, the PCR primers were evaluated by Primer-Blast tool on the NCBI site, www.ncbi.nlm.nih.gov/tools/primer-blast (Table 1).

cDNA synthesis

Onemicrogram of total RNA was used to synthesize the first strand complementary DNA (cDNA), using murine leukemia virus (MuLV) reverse transcriptase (Fermentas, Lithuania), in the presence of random hexamers and RNase inhibitor. The harvested cDNA was amplified by 35 cycles, using PCR Master Mix (Amplicon, Denmark). The products of PCR were tested by agarose gel (2%) electrophoresis and envisioned by ethidium bromide stain.

Quantitative real-time PCR

For Quantitative real-time RT-PCR (qRT-PCR), specific primers were designed for c-kit, STRA8 and PCNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control for normalizing the values. Reactions were performed, using SYBR® Premix Ex Taq™ II (Takara Bio Inc, Japan) on a Rotor-Gene™ 6000 real-time PCR machine (Corbett Research, Qiagen, Germany). Initial denaturation was performed at 95°C for 15 minutes, followed by 45 cycles of denaturation at 95 °C for 10 seconds, annealing under primer specific conditions, and extension at 72°C for 20 seconds. Comparative quantitation was performed between selected groups, using REST 2009 (Relative Expression Software Tool, Qiagen), based on PairWise Fixed Reallocation Randomization Test©.21. To assess the relative abundance of mRNA, mean CT values were calculated from triplicate reactions, ran for each sample. Relative gene expression was then calculated, using the Pfaffl method [34]. Then, the obtained data were evaluated, using one-way ANOVA, followed by Fisher's LSD post-hoc comparisons.

Statistical analysis

In this study, the Kolmogorov-Smirnov test was applied to check the normality, and then a parametric approach was used to analyze the data. The stereological data was compared, using one-way ANOVA. The

gene expression data obtained from Real-Time PCR was also analyzed by one-way ANOVA. All statistical work was performed in IBM SPSS statistics V21. Results were presented as mean \pm SD. The significance of comparisons was denoted by $p < 0.05$.

Results

Total volume of testis and interstitial tissue

The total volume of testis and interstitial tissue were significantly decreased in the mice, treated with busulfan, in comparison to the control and DMSO groups ($P < 0.001$). Transplantation with SCs did not ameliorate the reduction of testis volume, compared to the control and DMSO groups, but the volume of interstitial tissue increased in mice, after transplantation with SCs, in comparison with the busulfan groups ($P < 0.02$; Fig. 3 and 4).

Total length of seminiferous tubules

The total length of seminiferous tubules was significantly reduced in the mice, treated with busulfan, in comparison to the control and DMSO groups ($P < 0.01$). The total length of seminiferous tubules increased in mice, after transplantation with SCs, compared with the busulfan groups ($P < 0.05$; Fig. 3 and 4).

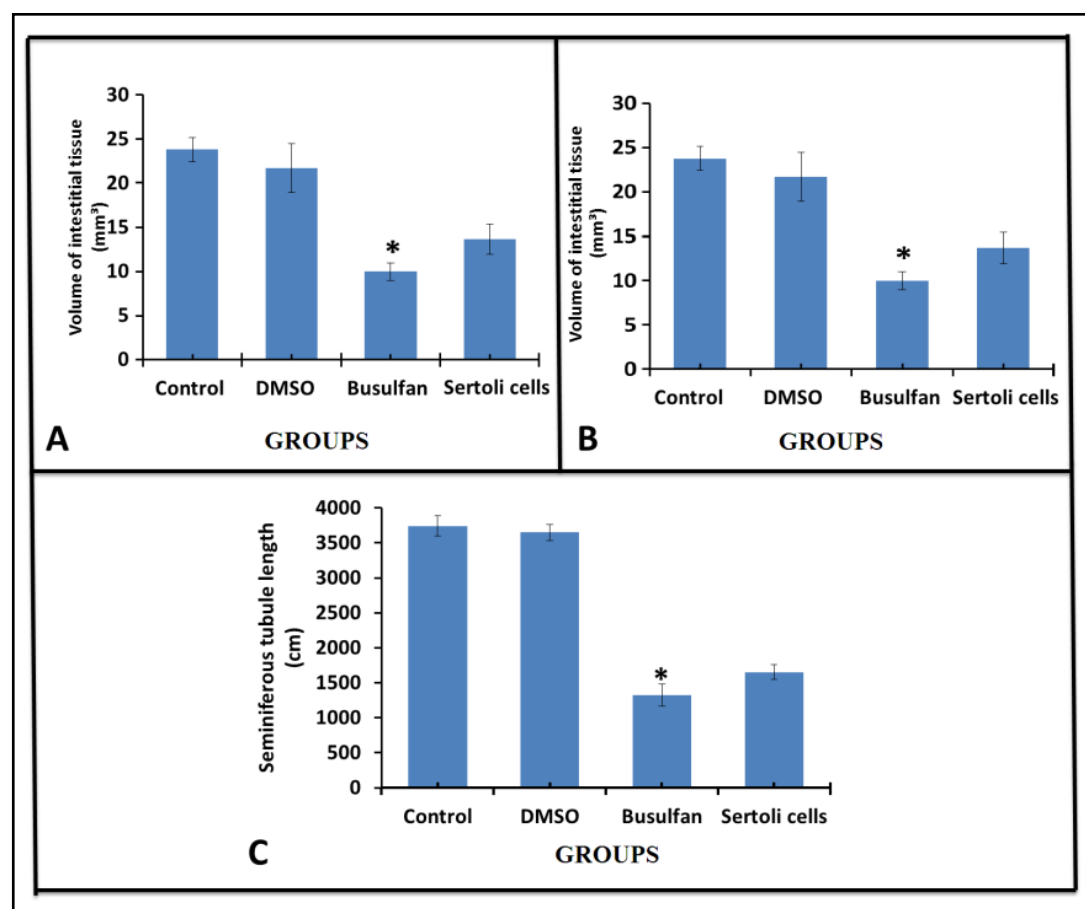


Fig. 3. (A, B) The total volume of testis and interstitial tissue in the different groups are shown. The significant difference between busulfan groups in comparison to the control and DMSO groups is indicated. * $p < 0.01$. (C) The total length of seminiferous tubules in the different groups are shown. The significant difference between busulfan groups in comparison to the control and DMSO groups is indicated. * $p < 0.01$.

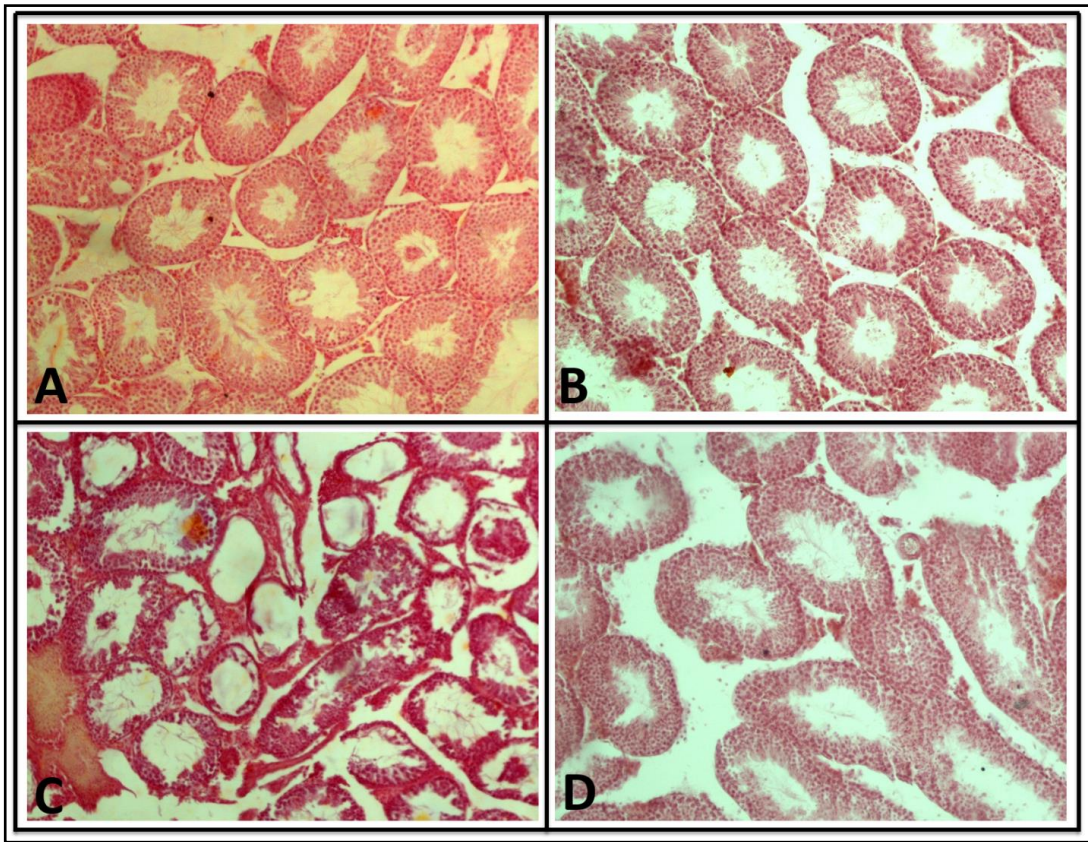
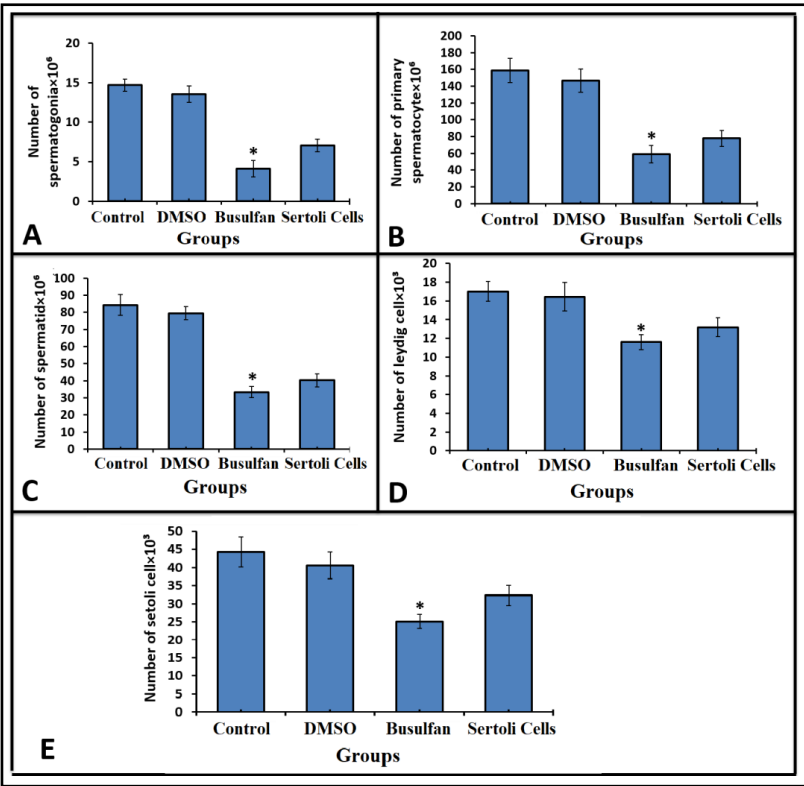


Fig. 4. Hematoxylin and eosin–stained histological Cross-sections of the seminiferous cords from Control groups (A), sham groups (B), 8 weeks after Busulfan injection (C), 8 weeks after Sertoli cells transplant (D).

Fig. 5. (A-E) The total number of spermatogonia, primary spermatocyte, round spermatid, Sertoli Cells and Leydig cells in the different groups are shown. The significant difference between busulfan groups in comparison to the control and DMSO groups is indicated. * $p < 0.01$.



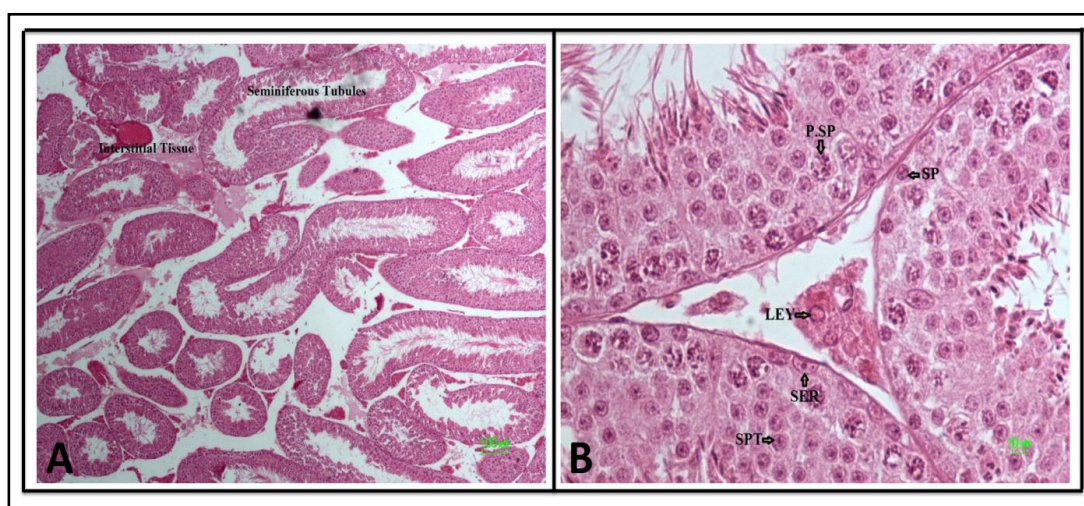


Fig. 6. Hematoxylin and eosin-stained histological Cross-sections of the seminiferous cords. Spermatogonia(SP), Primary spermatocyte (P. SP), Round spermatid (SPT), Sertoli cells (SER), Leydig cells (LEY).

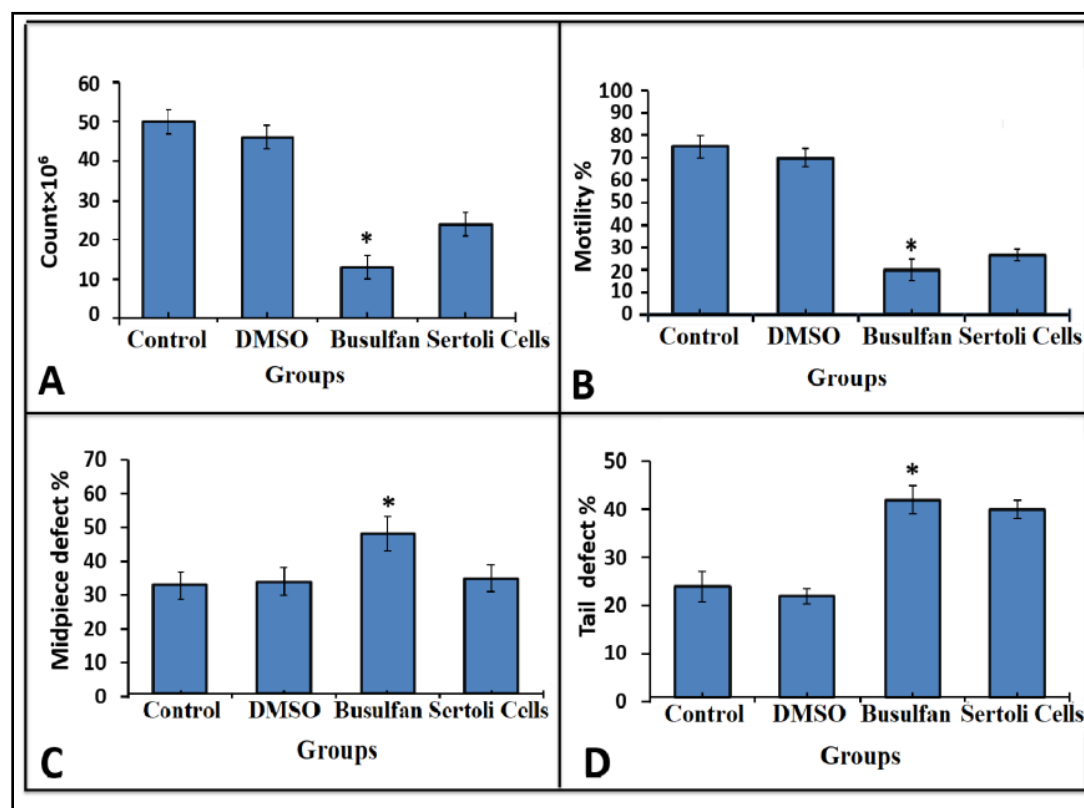


Fig. 7. (A-D) Total sperm count, motility and sperm mid-piece and tail defect in the different groups are shown. The significant difference between busulfan groups in comparison to the control and DMSO groups is indicated. * $p < 0.01$.

Number of Spermatogonia, primary spermatocyte, round spermatid, Sertoli Cells and Leydig cells

The total number of spermatogonia, primary spermatocyte, round spermatid, SCs and Leydig cells in busulfan-induced groups was decreased, compared to the DMSO, SCs and control groups ($P < 0.01$). Transplantation with SCs did not ameliorate the reduction of testis volume, in comparison with the control and DMSO groups, but the number of spermatogonia, primary spermatocyte, round spermatid, SCs and Leydig cells was increased, after transplantation of mice with SCs, in comparison with the busulfan groups ($P < 0.05$; Fig. 5 and 6).

Total sperm count, motility and sperm mid-piece and tail defect

Our data showed that the total sperm count in busulfan-induced animals was decreased, in comparison with the control, DMSO and SCs groups ($P < 0.001$). Busulfan produced an extreme significant reduction in sperm count in the busulfan groups, compared with the control and DMSO groups, but the sperm count after transplantation of mice with SCs showed significant improvement, in comparison with the busulfan groups ($P < 0.03$). Our data show that the sperm motility also decreased in the busulfan groups in comparison to the control, DMSO and SCs groups ($P < 0.002$). The SCs transplantation shows a significant improved sperm motility, in comparison with the busulfan groups ($P < 0.05$).

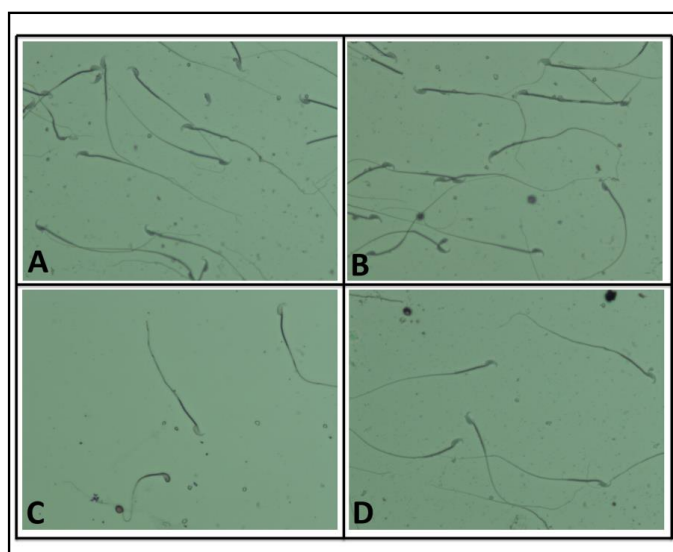


Fig. 8. Microscopic image of sperm sample was stained with Diff-Quik. (A) Control groups, (B) DMSO groups, (C) Busulfan groups, (D) Sertoli cells groups.

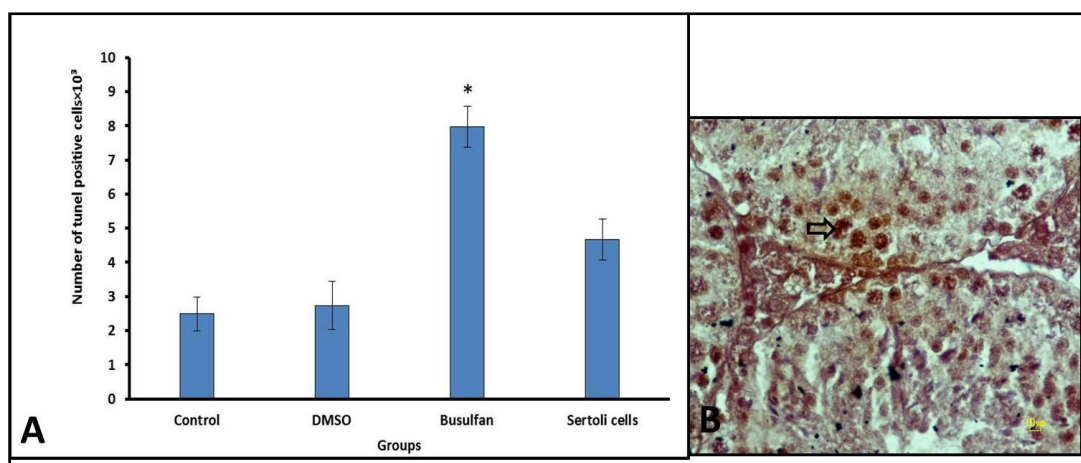


Fig. 9. (A) Total number of apoptotic cells in the different groups is shown. The significant difference between busulfan groups in comparison to the control and DMSO groups is indicated. * $p < 0.01$. (B) Representative microphotographs of TUNEL assay. TUNEL detection of apoptotic cells in testes. TUNEL-positive cells are stained in brown (Arrows).

The results showed a significant increase in the defects of the sperm mid-piece and tail in the busulfan groups, compared to the control, DMSO and SCs groups ($P < 0.001$). The SCs transplantation shows a significant reduction in sperm mid-piece and tail, in comparison with the busulfan groups ($P < 0.01$) (Fig. 7 and 8).

Increased Apoptosis in Testis

The effect of SCs transplantation on the apoptotic changes of spermatogenic cells was investigated by TUNEL assay. TUNEL-positive cell counts showed a significant increase in the apoptotic cells in the busulfan groups, in comparison with the control, DMSO and SCs groups ($P < 0.001$) (Fig. 9 and Fig. 10).

Expression levels of Ckit, STRA8 and PCNA

The relative mRNA expression levels of c-kit, STRA8 and PCNA between different groups were normalized by control, and quantitated. As shown in the figure below, the levels of c-kit and STRA8 expression in rat testicular tissue were significantly decreased at 8 weeks, after the last injection ($p < 0.001$), but there was not a distinct decrease for PCNA, compared to the control and DMSO groups ($P < 0.05$). The relative mRNA expression levels of the indicated genes (c-kit, STRA8 and PCNA) between groups were presented in the Fig. 11.

Discussion

SCs have the potency to discharge several types of factors, which play fundamental roles in regulating the self-renewal, multiplication and spermatogonial stem cell differentiation,

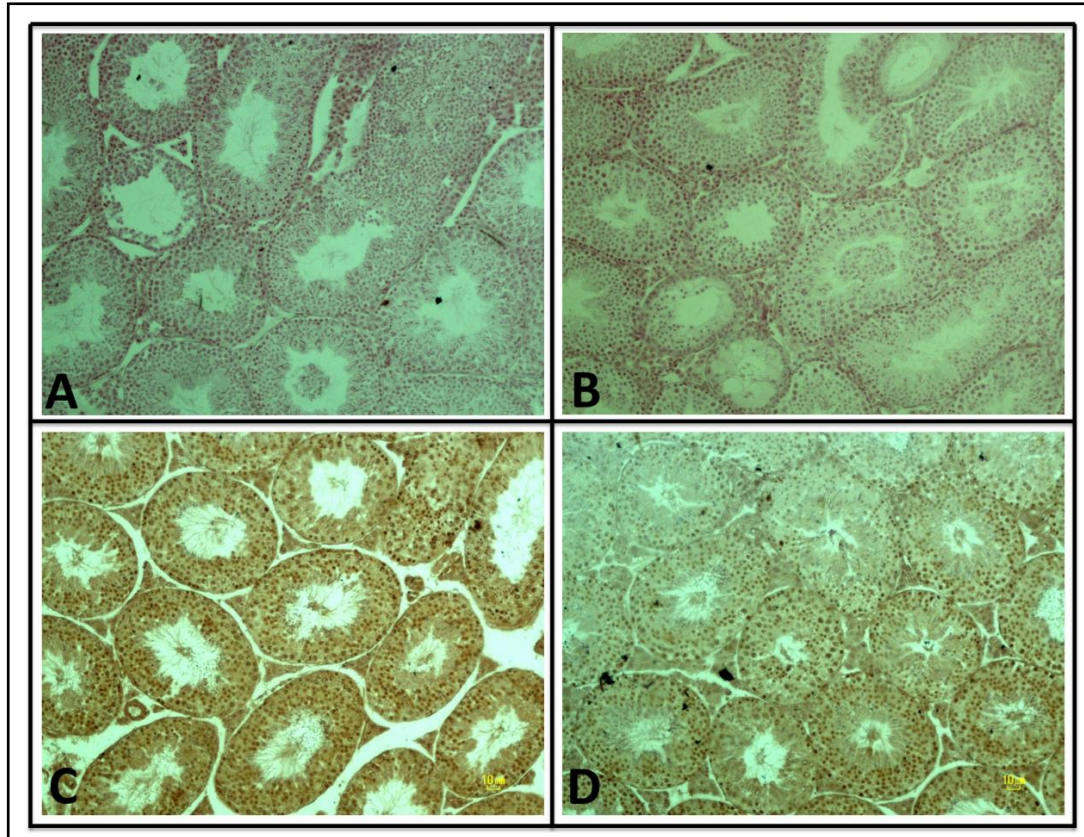
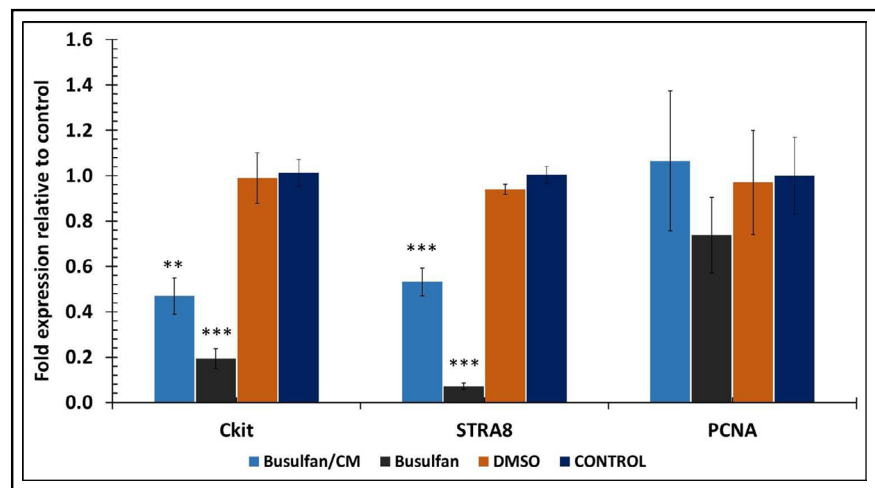


Fig. 10. TUNEL staining, histological Cross-sections of the seminiferous cords from Control groups (A), sham groups (B), after injection of Busulfan (C), after transplantation of Sertoli cells (D).

Fig. 11. The graph show the relative mRNA expression levels of c-kit (red line), STRA8 and PCNA between different groups in the testis tissue; the relative mRNA expression was tested at 8 weeks after the last injection, as shown in graph, the relative mRNA expression of c-kit and STRA8 in testis



tissue were decreased significantly after busulfan and busulfan/CM Injection compared to control and DMSO groups ($p < 0.000^{***}$), this decrease was more dominant in the busulfan groups than busulfan/CM groups, and also, the alterations of relative expression for PCNA wasn't significant between experimental groups in compared to DMSO and control groups ($^{***}p < 0.001$).

during the spermatogenesis process. Therefore, nowadays having a greater consciousness about the role of SCs transplantation in spermatogenesis, will be necessary for the control of male fertility by the homeostasis and regulation of SCs function.

In this study, we found that SCs transplantation could notably increases the sperm count and the number of testicular cells, such as spermatogonia, primary spermatocyte, round spermatid, SCs and Leydig cells. In addition, we also observed a remarkable enhancement in sperm motility, compared to the control, DMSO and busulfan groups, suggesting that SCs may regulate cell mitosis and meiosis of human male germ cells. Furthermore, we also investigated the relative mRNA expression levels of the indicated genes in testicular tissue between the groups, demonstrating a reduction or no significant difference at the transcriptional level of mice SCs. Likewise, we have seen the volume of interstitial tissue increases in mice after the transplantation with SCs. The previous reports stating the effect of SCs implanted, on the development of testicular interstitium and tubular structures, our observation of the potency of SCs was consistent with those of previous studies [35-38].

On the other hand, similar to our present report, recent observations propose an extraordinary role for the populations of interstitial cell in formation of spermatogonial stem cell, in specific, associative monitorings from previous studies [36-38] have shown that transplanted SCs, support spermatogenesis within the testis seminiferous tubules or in the interstitium of the testis or stimulates partial recovery of spermatogenesis [1, 35, 36, 39, 40-43]. In a study, Tesarik *et al.* indicated that short-term co-culture of germ cells with SCs could improve spermatogenesis [44], and another observation by Tajima *et al.* revealed that stimulating factors generated by SCs in co-culture system, could progress the spermatogonial stem cell differentiation [45]. In addition, Hue *et al.* utilized testis somatic cells for spermatogonial stem cell differentiation, and the germ cells could differentiate into spermatid *in vitro*. Their data revealed that SCs also stimulate differentiation in immortalized spermatogonial cell line [46]. Our findings consistent with previous studies, clearly demonstrate an improved *in vitro* differentiation in the presence of SCs. In parallel, Krawetz *et al.* (2009) expressed that the cytokines generated by SCs, play a significant role in the progression of spermatogonia and spermatocyte, the integrity of the immune system, and the function of the regulatory immune cells in the interstitium [47].

Conclusion

Taken together, our findings clearly demonstrated that SCs could ameliorate spermatogonia cell proliferation and differentiation *in vitro*, through secretion of growth factors. SCs transplantation may be beneficial as an approach and new therapeutic strategies, for male infertility in reproductive and regenerative medicine. These cells can provide fundamental sources of different types of male germ cells and SCs, contributing to the determination of the genetic and epigenetic regulators, in controlling the spermatogonia mitosis and spermatocytes meiosis.

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Disclosure Statement

The authors declare to have no competing interests.

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