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Mesenchymal Stem Cells for Busulfan–Induced Azoospermia: An Experimental Study

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ABSTRACT

Background: Busulfan is a frequently used chemotherapeutic agent. It exerts harmful effects on male reproduction. Azoospermia is a major cause of male infertility, and stem cell therapy may be a reasonable treatment option.

The Aim of The Work: The present study aimed to examine the potential role of mesenchymal stem cell transplantation for treatment of busulfan–induced azoospermia.

Patients and Methods: A total of 45 mice were used and divided equally into three groups; the control groups that received none and used for provision of bone marrow mesenchymal stem cells. Other 30 received intraperitoneal busulfan [10 mg/kg] in two doses of with 21 days interval to induce azoospermia. Then 15 received stem cell transplantation 35 days after last busulfan injection. Animals were followed for 35 days, with blood cell count, measurement of testosterone levels and oxidative stress markers and testes were examined for histomorphometric changes.

Results: Body weight in busulfan group was significantly increased than the stem cells group [35.93±1.39 vs 26.87±1.18 respectively], but it still lower than the control group. The luminal diameter of seminiferous tubules was significantly increased in the busulfan than the control or stem cells-treated groups [148.0±8.8 vs 58.0±10.8 and 115.3±9.2 respectively]. All blood cells were reduced by busulfan injection and partially regained by stem cell therapy. Spermatogenic potential was significantly reduced in busulfan than the control group [0.6667±0.487 vs 6.60±0.507]. It was significantly regained by stem cell therapy, but still lower than control group [5.53±0.516 vs 6.60±0.507]. Testosterone, glutathione reductase, superoxide dismutase and glutathione peroxidase were significantly reduced, while lactate dehydratase and malondialdehyde were increased by busulfan and partially regained by stem cell therapy, with significant differences between groups.

Conclusion: Busulfan-induced azoospermia and harmful effects on male mouse reproduction system and it mainly affected spermatogenic potential. These effects could be restored by mesenchymal stem cell transplantation.

Keywords: Azoospermia; Busulfan; Mesenchymal Stem Cells; Redex.

INTRODUCTION

Infertility represents an important and challenging health problem as it affects up to 12% [8-12%] of couples worldwide. About half of infertility is due to a male factor [primary or contributing factors] with reproductive problems in males responsible for the development of infertility. The male infertility is due to the failure of germ cells proliferation and differentiation or due to somatic-cell dysfunction [1].

Assisted reproductive techniques are the management for male factor infertility. However, azoospermia or sperm deformity has been a challenge for reproductive medicine. In addition, patients received chemotherapy for cancer are at increased risk for reproductive failure due to the adverse effect of drugs. Thus, stem cell therapy has attracted significant interest **[2].** The rationale for introduction of stem cells in management of azoospermia depends on the identification of the high capability of stem cells to produce different types of cells. Stem cells are undifferentiated cells with two main characteristics-self-renewal and the ability to differentiate into more specialized cell types **[3].** Mesenchymal stem cells [MSCs] are a group of pluripotent adult stem cells. They can be easily isolated from a small primary tissue sample such as bone marrow [BM], fat tissue, blood samples, and amniotic fluid. They are highly proliferative cells **[4].**

Although, the research in use of stem cells in treatment of male factor infertility, contradictory results were reported. Some studies have shown that under optimal conditions and with proper enhancers, stem cells had the ability to differentiate into male germinal cells [GCs] **[5,6].** Others believe that stem cells did not impact renewal of testicular gametogenesis in infertile animal models **[7,8],** and other studies have shown that stem cells positively affect reconstruction of germinal epithelium and restoration of fertility **[9-12].**

THE AIM OF THE WORK

The present study was designed to examine the role of mesenchymal stem cell transplantation for treatment of Busulfan–induced azoospermia.

METHODOLOGY

The present work included sexually mature, 6-weeks old, 45 male mice, weighing 25-35 g. They were housed in a temperature controlled room with 12 hours day-light cycles. The rats were fed with standard commercial chow diet ad libitum and had free access to water. They were kept in their environment for two weeks for adaptation before the start of the trial. They were divided into three groups [15 mice each]: the busufen-induced azoospermic group, the control group and busulfan plus stem cells group. The control group was applied as stem cell donors.

Isolation of BM-MSCs: Mice of control group were decapitated under anesthesia by IP injection of 100 mg/kg ketamine and 7 mg/kg xylazine. Incision was made on the skin and both femurs and their muscular tissues were completely removed. BM-MSCs were isolated from the femurs. Under sterile conditions, both ends of the bone were cut and the bone marrow was flushed out using an insulin syringe filled with Dulbecco's modified eagle medium [DMEM] supplemented with 1% penicillin streptomycin. Then, cells were extracted, cultured and BM-MSCs were isolated as described by Asadi-Yousefabad *et al*. [13].

Busulfan-induction of azoospermia: Male Mice were induced azoospermia by 10 mg/kg intraperitoneally injection of two doses of busulfan [Busilvex®, Pierre Fabre Medicament, Boulogne, France] with 21 days interval.

Bone marrow- mesenchymal stem cells [BM-MSCs] transplantation: It was performed 35 days after the last injection of busulfan. The busulfan treated animals were anesthetized. The suspension of BM-MSCs [10^6 cells in 100µL] was injected into the testes. Five weeks after transplantation, testes were removed and examined.

Body and testes weight analysis: The mice were weighed, and both testes were collected and weighted using a Precision Electronic Scale [ES320; D&T, Tianjin, China].

Blood analysis: Peripheral blood samples were collected from the orbital sinus of anesthetized recipient mice at days 14, 21, 28, and 35 according to Hoff's method [14] after stem cell transplantation. Blood samples of approximately 30 µL were collected from each mouse in 2 mL diluent [MEK-6318K, Nihon Kohden, Japan] to prevent clotting of the blood. Then, different blood cells were counted and hemoglobin concentration was assessed by an Automatic Hematology Analyzer MEK-6318K [Nihon Kohden, Tokyo, Japan]. In addition, the blood samples were centrifuged and serum was used for ELISA testing for the testosterone, lactate dehydrogenase [LDH], malondi-aldehyde [MDA], and glutathione reductase levels. We used ELISA kit [Cayman Chemical, USA]. 50 µl of serum was prepared and incubated for 30 minutes at 37 °C; then, the samples were washed for 10 seconds five times, and 50 µl of HRP-conjugate reagent was added and incubated with the cells for other 60 minutes at 37 °C. Then, the wells were washed for 10 seconds five times and incubated for another 30 minutes at 37 °C with 50 µl of a mixture of substrate A and B solutions; finally, 50 µl of stop solution was added to the wells. Ultimately, the light absorbance was detected by using a spectrophotometer [BioTek, USA] and values were determined against standard curve provided by the manufacturer.

Sperm count and motility: At the time of dissection, 1 cm of distal end of the vas deferens of each tests, was removed and preserved in 5 mL of Hank's balance salt solution [HBSS] at room temperature. A diffusion method was used to collect sperms as described by Seed *et al*. [15]. Sperm count was carried out after 10 minutes using a hemocytometer [16]. Total sperm count was calculated using the formula: A=B×C×D where A is the total number of sperm per 1 mL of semen, B is the total number of

sperm calculated per 0.1 CC of solution, C is the depth factor and D is the dilution factor [= 5 mL].

Histomorphometric analysis of testes: At the end of the study, animals were decapitated under anesthesia and their testes were removed and fixed in a 10% formalin buffer solution. Ethanol and xylene were used for dehydration and paraffin sections were prepared at a thicknesses of 5 µm. For each testis, five vertical sections from the polar and the equatorial regions were sampled. Then hematoxylin-eosin staining was performed and sections were examined. Then for evaluation of histomorphometric indices, in 10 circular transverse sections of tubules, outer, inner, and total diameters were measured according to Panahi *et al.* [17]. The testes were also rated for its spermatogenic potential according to the modified spermatogenic index on a modified scale of 0 to 7 according to Panahi *et al.* [17].

Statistical analysis: The collected data were fed to a personal computer through an excel sheet. Then, transferred to the statistical package of social sciences, version 18 [IBM®SPSS® Inc., Chicago, Armonk, USA]. All data were expressed by their mean [measure of central tendency] and standard deviation [measure of dispersion]. Groups were compared by one way analysis of variance [ANOVA] test, and paired group comparison was completed by the post Hoc least significant differences [LSD]. P value < 0.05 was set as the measure of statistical significance.

RESULTS

Fourteen days after injection of busulfan, the body weight of the mice did not differ significantly between studied groups. However, at the end of the study [35 days after injection], the total body weight in busulfan-treated group was significantly increased when compared to group treated by stem cells [35.93±1.39 vs 26.87±1.18 respectively]. However it was significantly lower than the control group. The weight of the testes in busulfan induced azoospermia was significantly lower than the control or stem cells-treated group [30.33±5.16 vs 120.67±7.52 and 57.33±8.20 mg, respectively]. These data indicated that busulfan treatment was associated with significant reduction of the body and testicular weight; the action was partially reversed by stem cells [Table 1].

In the current work, the luminal diameter of seminiferous tubules revealed that, it was significantly increased in the busulfan induced azoospermic group when compared to control group or stem cells-treated group [148.0±8.8 vs 58.0±10.8 and 115.3±9.2 respectively]. Luminal area on the other hand, significantly increased in busulfan-treated group when compared to control group. Stem cells treatment associated with significant reduction of luminal area. However, it remains higher than the control group. Cellular diameters, and cellular area significantly reduced by busulfan treatment, the action which reversed by stem cell therapy, but still significantly different than control group. Total diameter and cross section area significantly increased by busulfan than the control group and stem cells treated groups [Table 2]. Blood cell analysis showed significant variances between studied groups for red blood cell count, white blood cell count, hemoglobin concentration and platelet count. Busulfan treatment was associated with significant reduction of all cells of the blood and hemoglobin concentrations. The effect was partially reversed by stem cell therapy [Table 3]. Spermatogenic potential was significantly reduced by busulfan treatment when compared to control group [0.6667±0.487 vs 6.60±0.507 respectively]. It was significantly reversed by stem cell therapy, but still reduced than control group [5.53±0.516 vs 6.60±0.507 respectively]. Sperm count was significantly higher in the control than stem cell treated group. Similarly, testosterone, glutathione reductase, superoxide dismutase and glutathione peroxidase were significantly reduced by busulfan and partially regained by stem cell therapy, but to value significantly lower than control group. On the other side, LDH and MDA activity were significantly increased by busulfan treatment and reduced by stem cell therapy to values near that of the control group with significant differences [Table 4]. Figures [1] to [3] showed the different histopathological changes induced by busulfan that were partially restored by stem cell therapy.

Table [1]: Comparison between study groups regarding weight of the tests, and body weight at the start of the study and at the end of it.

Variables	Measures	Study group [treated]	Positive control [induced azoo]	Negative control [non-ttt, non-induced]	F	p
Weight of the tests [mg]	Mean±SD	57.33±8.20	30.33±5.16$	120.67±7.52#	641.5	<0.001*
Initial Weight [g]	Mean±SD	30.13±2.77	30.07±1.98	30.20±2.04	0.13	0.98
Final Weight [g]	Mean±SD	26.87±1.18	35.93±1.39$	39.07±1.39#	22.15	<0.001*

Significant difference when compared to the study or positive control groups; $ significant when compared to the study group

Table [2]: Seminiferous tubular data, among the study groups

	Study [stem cells]	Induced azoospermia [Positive control]	Negative control [no treatment]	F	p
Luminal diameter [µm]	115.3±9.2	148.0±8.8$	58.0±10.8#	335.02	<0.001*
Luminal area x 10^3 µm²	14.3±1.4	16.3±1.9$	3.9±0.7#	333.4	<0.001*
Cellular diameter [µm]	62.7±2.5	28.2±2.1$	51.1±3.2#	656.2	<0.001*
Cellular area x 10^3 µm²	14.3±1.0	2.9±0.8$	9.8±1.1#	537.4	<0.001*
Total diameter [µm]	246.7±14.0	204.0±9.3$	153.3±11.8#	234.1	<0.001*
Cross sectional area x 10^3 µm²	48.5±3.3	35.7±2.3$	24.1±2.3#	310.0	<0.001*

Significant difference when compared to the study or positive control groups; $ significant when compared to the study group

Table [3]: Changes of blood cell indices over the study period

		Study [stem cells]		Induced azoospermia [Positive control]		Negative control [no treatment]		F	p
		Mean	S.D	Mean	S.D	Mean	S. D		
Red blood cell count x 10^6/ml	At 14 days	6.5467	0.18074	4.8800$	0.28835	6.6933	0.36148	185.235	<0.001*
	At 21 days	6.4867	0.21668	4.6133$	0.33989	6.2400#	0.35416	161.935	<0.001*
	At 28 days	6.1933	0.13345	5.0467$	0.35024	6.6333#	0.39400	102.108	<0.001*
	At 35 days	6.6333	0.20237	5.8467$	0.39617	6.7333	0.39761	29.811	<0.001*
White blood cells count x 10^3	At 14 days	11.8467	0.43238	8.6267$	0.84808	13.0867#	1.29993	91.865	<0.001*
	At 21 days	11.3267	0.52843	8.8533$	0.78546	12.5467#	1.21530	67.147	<0.001*
	At 28 days	10.8733	0.52978	9.2067$	0.94602	12.7200#	1.30778	48.160	<0.001*
	At 35 days	10.5467	0.68647	9.5867$	1.02321	12.0200#	1.00513	26.736	<0.001*
Hemoglobin [mg/dl]	At 14 days	11.8333	0.51640	9.8200$	0.37645	12.1533	0.57429	97.539	<0.001*
	At 21 days	12.4067	0.40790	10.0933$	0.44476	11.6533#	0.50831	100.622	<0.001*
	At 28 days	11.9333	0.34983	10.6733$	0.41827	11.9333	0.36580	55.235	<0.001*
	At 35 days	12.2867	0.33989	11.3533$	0.41034	11.7733#	0.34531	24.390	<0.001*
Platelet count x 10^3	At 14 days	449.0000	23.35135	278.9333$	22.83314	474.8667#	42.64616	176.708	<0.001*
	At 21 days	419.8667	19.83455	238.0000$	20.85665	519.2667#	40.98165	365.046	<0.001*
	At 28 days	488.5333	20.49344	297.2667$	19.02805	490.8000	11.12398	613.082	<0.001*
	At 35 days	478.6667	18.94227	295.2000$	26.72131	436.0667#	28.21972	221.961	<0.001*

Table [4]: Comparison between study groups regarding spermatogenic potential, sperm count, testosterone and oxidative stress markers

	Study [stem cells]		Induced azoospermia [Positive control]		Negative control [no treatment]		F	P
	Mean	S. D	Mean	S. D	Mean	S. D		
Spermatogenic potential	5.5333	0.51640	0.6667$	0.48795	6.6000#	0.50709	590.888	<0.001*
Sperm count x 10^6	23.8000	4.69346			34.8667	7.22957	24.72	<0.001*
Testosterone [ng/ml]	0.5200	0.10142	0.1733$	0.08837	0.6200#	0.11464	79.155	<0.001*
LDH	107.9333	8.10173	213.7333$	14.59680	99.2667#	6.82921	561.824	<0.001*
MDA activity	1.7600	0.10556	3.6600$&	0.22615	1.6667	0.20587	544.019	<0.001*
Glutathione reductase	12.7333	1.53375	3.8000$	1.08233	9.8667#	1.12546	195.429	<0.001*
SOD	27.2667	2.37447	11.1333$	2.16685	31.0667#	3.32666	235.536	<0.001*
Glutathione peroxidase	17.4000	2.47271	5.3333$	1.34519	21.4667#	2.50333	223.262	<0.001*

Significant difference when compared to the study or positive control groups' $ significant when compared to the study group; & significant when compared to negative control group.

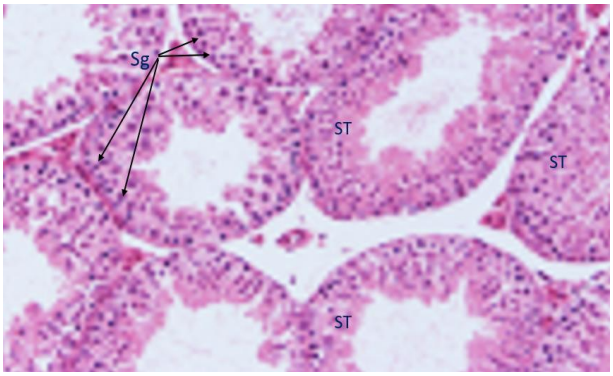

Figure (1): Photomicrograph of the control group tests (non-treated group), showing normal architecture and shape of seminiferous tubules (ST). Spermatogonia (Sg) were normally arranged with intact basement membrane [H and E x 100].

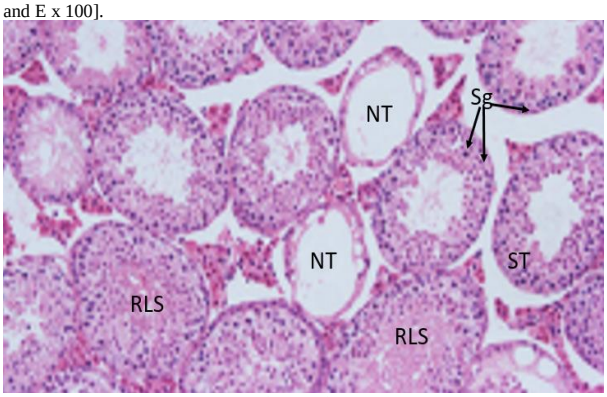

Figure (3): Photomicrographs of busulfan plus stem cells treated group, demonstrated that, restoring normal structures of seminiferous tubules (ST) with normal spermatogonia arrangement (Sp) and some tubules still necrotic (NT) with acellular structure (empty) and others filled by round and elongated spermatocytes (RLS). H & E x 100.

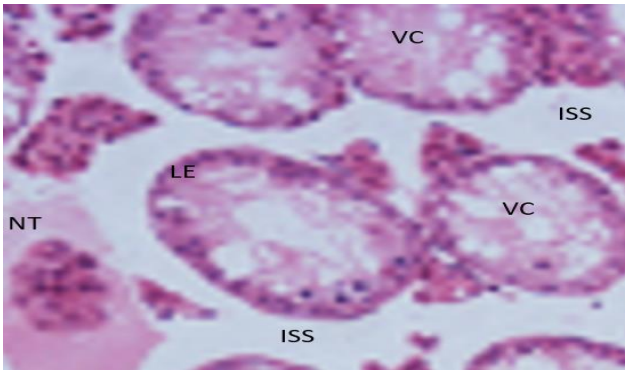

Figure (2): Photomicrograph of busulfan treated group, showed thinner lining epithelium (LE), vacuolated cytoplasm of the lumen and presence of some necrotic tubules (NT); the interstitial spaces (ISS) were widened indicating shrinkage of tubules. The overall structures are hazy [H and E x100].

DISCUSSION

The results of the current work showed that, stem cell transplantation was associated with spermatogenic potential restoration in a busulfan-induced azoospermia in a mouse model. The harmful effects of busulfan seems to be due to multiple mechanisms [e.g., hormonal imbalance and oxidative stress]. Histologically, busulfan treatment was associated with increased luminal diameter and vacuolations of seminiferous tubules, and significant reduction of spermatogenic cells and healthy sperms. These changes were reversed by stem cell therapy. These results confirm results of the Qian *et al*. [18] study.

In the current work, busulfan-treated mice had significantly lower levels of serum testosterone. These results are in line with Mohammad-Ghasemi *et al*. [19] and Bordbar *et al*. [20] who reported that, testosterone was significantly dropped after intraperitoneal injection of busulfan in a male mice. However, such results could not be obtained by Dehghani *et al*. [21] who reported that, testosterone did not significantly changed after intraperitoneal injection of busulfan in a male mice model, irrespective of their documentation of other harmful effects of busulfan on the testes.

Serum testosterone levels were significantly increased after stem cell transplantation. These results are in line with that of Hassan and Alam [22] who reported that, the injection of stem cells was used to manage chemical-induced male infertility in a rat model and associated with significant restoration of serum testosterone levels.

The current results are also in accordance with Anand *et al*. [23] who used adipose-derived mesenchymal stem cells to treat busulfan-induced azoospermia in a rat model and reported a restoration of testicular size and weight with stem cell therapy.

Hsiao *et al*. [24] reported that, stem cell therapy was associated by significant improvement of sperm count and percentage of live forms and progressive motility in a rat model of testicular torsion.

Qian *et al*. [18] also reported that, the stem cell transplantation in a mouse model of busulfan-induced azoospermia was associated with significant increase in the total number of sperms and percentage of motile and normal forms. In addition, it inhibited the percentage of abnormal forms of sperms.

Previous results of restoration of testicular size and weight, with improved testosterone levels and spermatogenic potential; all indicated that stem cell therapy effectively regain endogenous spermatogenesis which was impaired by busulfan.

In addition, results of the current study showed significant reduction of anti-oxidant enzymes and significant increase of lipid peroxidation on testicular tissues. The harmful effects of busulfan could be attributed to its direct toxic action on the testicular tissues, with its general non-specific actions as reduction of serum testosterone levels, as reported by Ilbey *et al*. [25].

The increased oxidative stress in the current study was reflected by significant increase of malondialdehyde levels with concomitant reduction of protective antioxidant activities of superoxide dismutase and glutathione peroxidase. Nasimi *et al*. [26] reported similar results. Interestingly, this oxidative stress on the testes could lead to significant reduction of serum testosterone and testicular damage [21].

In line with the current results, Qin *et al*. [27] reported that, intraperitoneal injection of busulfan damages the hematopoiesis function with severe side effects in animal models. They added that, doses above 28mg/dl is associated with permanent damage of testicular function and fertility could not be restored. The lower doses of busulfan used to induce azoospermia could be responsible for the reversible damage of testicular tissues, hormonal changes and blood cell count restoration to value near that of the control group.

Conclusion:

Busulfan-induced azoospermia and harmful effects on male mouse reproduction system and it mainly affected spermatogenic potential. These effects could be restored by mesenchymal stem cell therapy.

Conflicts of interest

All authors declare that they have no specific conflicts of interest.

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