The radiation protection and therapy effects of mesenchymal stem cells in mice with acute radiation injury

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ABSTRACT. The aim of this study was to investigate the effects and mechanisms of mesenchymal stem cells (MSCs) on haematopoietic reconstitution in reducing bone marrow cell apoptosis effects in irradiated mice, and to research the safe and effective dosage of MSCs in mice with total body irradiation (TBI). After BALB/c mice were irradiated with 5.5 Gy cobalt-60 γ-rays, the following were observed: peripheral blood cell count, apoptosis rate, cell cycle, colony-forming unit-granulocyte macrophage (CFU-GM) and colony-forming unit-fibroblast (CFU-F) counts of bone marrow cells and pathological changes in the medulla. The survival of mice infused with three doses of MSCs after 8.0 Gy or 10 Gy TBI was examined. The blood cells recovered rapidly in the MSC groups. The apoptotic ratio of bone marrow cells in the control group was higher at 24 h after radiation. A lower ratio of G0/G1 cell cycle phases and a higher ratio of G2/M and S phases, as well as a greater number of haematopoietic islands and megalokaryocytes in the bone marrow, were observed in the MSC-treated groups. MSCs induced recovery of CFU-GM and CFU-GM and improved the survival of mice after 8 Gy TBI, but $1.5 \times 10^8 \text{ kg}^{-1}$ of MSCs increased mortality. These results indicate that MSCs protected and treated irradiated mice by inducing haematopoiesis and reducing apoptosis. MSCs may be a succedaneous or intensive method of haematopoietic stem cell transplantation under certain radiation dosages, and could provide a valuable strategy for acute radiation syndrome.

Currently, substantial progress has been made in the treatment of acute radiation syndrome (ARS) [1–6]. The study of haematopoietic growth factors and haematopoietic stem cell transplantation (SCT) has already progressed to the molecular and genetic level [4, 7, 8]. However, there are still some problems associated with SCT for ARS, such as donor deficiency, a high mortality for conditioning and complications resulting from transplantation. Thus, some experts continue to question the effect of transplantation on ARS. It is known that the microenvironment in bone marrow plays a key role in haematopoietic recovery after irradiation injury. The haematopoietic stem cells (HSCs) and the microenvironment, in a pathological state, often damage and aggravate mutually [9]. Therefore, the treatment of both is indispensable in ARS. Mesenchymal stem cells (MSCs) are adherent cells capable of self renewal and multilineage differentiation [10]. In addition, MSCs can produce several essential haematopoietic growth factors, including interleukin (IL)-6, IL-11, leukemia inhibitor factor (LIF), stem cell factor (SCF), Flt3 ligand and stromal derived factor (SDF) [11]. The microenvironment in bone marrow consists of stroma cells derived from MSCs. Cytokine and extracellular matrices are important factors in HSC growth and multiplication [12]. Transplantation of MSCs is used for both autologous and allogeneic transplant [13, 14], but there are fewer reports on the radiation protection and therapy effects of MSCs on mice with acute radiation injury [15, 16]. The present study was undertaken to examine the effects of three dosages of MSCs on the survival of mice exposed to lethal doses of total body irradiation (TBI), and to explore the mechanisms by which MSCs significantly improve haematopoietic recovery. This was accomplished by observing peripheral blood counts, apoptosis, p53 expression, CFU-GM and CFU-F counts, the cell cycle of bone marrow cells, and pathological changes in variety of the medulla.

Methods and materials

Mice

MSC donor mice (male C57BL/6) and bone marrow cell (BMC) donor mice and recipients (female BALB/C) 6–7 weeks of age were purchased from the Experimental Animal Facility of the Academy of Military Medicine Sciences, China. Mice were housed five to a cage and received commercial rodent chow and autoclaved acidified water. All studies were performed in accordance with protocols approved by the Institutional Care Committee.

Isolation and culture expansion of MSCs

Femurs were taken from donor mice killed by cervical dislocation under CO_2 anaesthesia. Mononuclear cells

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were separated by centrifugation at $1000 \times g$ for 10 min at room temperature on a percoll gradient (density, 1.082 g ml^{-1}). The interface was collected and resuspended in phosphate buffered saline (PBS). The cells were suspended in Dulbecco's Modified Eagle Medium-low glucose (DMEM-LG), supplemented with 10% foetal bovine serum (FBS) and 10 ng ml^{-1} epidermal growth factor (EGF); $1 \times 10^6 \text{ cells cm}^{-2}$ were plated in culture flasks (Hyclone, Logan, UT). The culture was maintained at 37°C in a humidified environment containing 5% CO_2. After 48 h, the non-adherent cells were removed and the medium replaced. When 80–90% confluence was reached, adherent cells were trypsinised with 0.02% trypsin (Hyclone, Logan, UT) at 37°C for 5 min.

Phenotype of MSCs

The harvested cells were washed and resuspended in PBS in aliquots of 1×10^6 cells. The cells were stained with fluorescein-conjugated antibody against CD34, CD45, CD105, CD29 and Sca-1 (PharMingen, San Diego, CA) [12, 17] and incubated for 30 min in the dark at room temperature (1 μg antibody per 10^6 cells). After being washed and resuspended, the cells were analysed in a flow cytometer using cell Quest software (Becton-Dickinson, San Jose, CA).

Differentiative ability of MSCs

For osteogenic differentiation, MSCs were plated in induction media (DMEM, 10% FBS, 10 mM β-glycerophosphoric acid, 50 μM vitamin C, 0.25 μM dexamethasone) in a density of 5000 cells cm^{-2}, cultured for 3 weeks and then stained for the presence of mineral with the von Kossa method. The cells were cultured in adipogenic induction media (DMEM, 10% FBS, 0.1 μM dexamethasone, 50 μM indomethacin and 0.5 mM 1-methyl-3-isobutylxanthine) with a density of 10^4 cells cm^{-2}. The culture medium was replaced every 2 days and stained with oil red O solution to show lipids in adipose cells.

Radiation

Recipient mice were irradiated using a cobalt-60 source at a dose rate of $3.99 \times 10^{-2} \text{ C kg}^{-1} \text{ min}^{-1}$. For haematopoietic studies, TBI was performed as a single exposure of 5.5 Gy [18]. $2.5 \times 10^7 \text{ kg}^{-1}$ of MSCs were infused via the tail vein 4 h after irradiation. Control mice were injected with a comparable volume of PBS.

For survival studies, recipient BALB/c mice were transplanted with $2.5 \times 10^7 \text{ kg}^{-1}$, $5 \times 10^7 \text{ kg}^{-1}$ or $1.5 \times 10^8 \text{ kg}^{-1}$ of MSCs after 8 Gy TBI, while being co-transplanted with $1 \times 10^9 \text{ kg}^{-1}$ of BMCs of donor mice. Control mice were only infused with $1 \times 10^9 \text{ kg}^{-1}$ of BMCs.

MSCs – engraftment detection

The *sry* gene of the Y chromosome in the BMCs of recipient female mice were detected by polymerase chain reaction (PCR) on days 7 and 30. The primer sequence of the Y chromosome forward primer was 5′-ATTTATG GTGTGGTCCCG-3′, and the reverse primer 5′-GCTGTA AAATGCCACTCC-3′ (Bioasia, SangHai, China). The PCR conditions were: 36 cycles at 95°C for 50 s, 55°C for 60 s, 72°C for 60 s, followed by 1 cycle at 72°C for 10 min [19, 20].

Peripheral blood counts

Each mouse was bled by retro-orbital puncture for blood cell counts every 3–4 days. Blood (20 μl) was collected in a vial containing 1 μl of 0.5 M ethylenediaminetetraacetic acid. Blood cell counts were performed automatically on an F-820 vet haematology analyser (Sysmex, Japan).

CFU-GM and CFU-F assay

At days 4, 12 and 25, cells were brushed out from the femur. 2×10^5 mononuclear cells were plated per well using six-well tissue culture plates. The media was composed of α-MEM (minimum essential medium) supplemented with 20% horse serum, 100 ng ml^{-1} granulocyte monocyte colony stimulating factor (GM-CSF) and 3% L-glutamine. CFU-GM were counted at day 7 of the culture at 37°C in a humidified environment containing 5% CO_2, with colonies consisting of at least 50 cells. Bone marrow cells were plated at 1×10^6 in RPMI 1640 media including 10% FBS and $10^{-6} \text{ mol l}^{-1}$ hydrocortisone. The fibroblast colonies were counted using an inverted microscope at $\times 25$ on day 14. Cell clusters consisting of at least 40 fibroblasts were scored as a CFU-F colony.

Cell-cycle and apoptosis ratio and pathological changes in the bone marrow

After irradiation, the myelocyte count was taken and adjusted to a density of 5×10^5 cells. The cells were washed twice with PBS, resuspended with 5% calf blood serum PBS (0.3 ml), added to 0.7 ml anhydrous alcohol and mixed at -20°C for 24 h, before being added to 200 μl (1 mg ml^{-1}) of RNA enzyme at 37°C for 30 min and then 100 μg ml^{-1} propidium iodide (PI) dyes (300 μl) for 20 min. Finally, the sample was measured with flow cytometry. The mice sternum section was observed using haematoxylin and eosin stain at days 12 and 23.

Statistical analyses

Results of replicate experiments were represented as the mean $\pm$ standard deviation (SD). Each data point represents 5–10 mice. Data were analysed by a *t*-test for haematological data and by a Kaplan–Meier analysis for survival experiments with SAS 9.0 software.

Results

Identification and engraftment of MSCs

MSCs have a spindle-shaped fibroblastic appearance and are positive for CD29, CD44, CD105 and Sca-1. No

positive cells for the haematopoietic antigens CD34 or CD45 could be detected by flow cytometry. Adipogenic differentiation was shown by the accumulation of lipid vacuoles, which were stained by oil red O. Osteogenic differentiation was indicated by calcium deposition seen with the von Kossa method. The positive expression of *Sry* genes of MSCs was detected in the bone marrow of recipient female mice on days 7 and 30 (Figure 1).

Peripheral blood assay

The number of white blood cells (WBCs) was always significantly higher in the initial stage, nadir stage and recovery stage of ARS in the MSC-treated groups than in the control group ($p < 0.05$). The WBC count in the MSC groups dropped to a nadir that was 20% of the pre-irradiation count on day 17. WBC recovery accelerated after 17 days and recovered to 90% of the pre-irradiation level on day 26. By contrast, WBC counts in the control group dropped until the nadir was about 17% of its original level. After day 17, counts slowly increased and only recovered to 80% of the original amount on day 26 (Figure 2). The haemoglobin count in the two groups showed no statistically significant difference. It dropped to the nadir and began to recover almost on the same day. Normal levels were reached at day 22 (Figure 3). The platelet count was slightly higher in the MSC-treated groups than in the control group in the initial and nadir stages. In the recovery stage, the counts were significantly increased and recovered to the pre-irradiation level at day 23 in the MSC group. The count in the control group only reached 60% at day 23. The recovery still lingered at 80% and recovery was not complete at day 30 (Figure 4).

CFU-GM and CFU-F assay

The percentage of CFU-GM was 12 ± 4.8 in the MSC group on day 4, 24 ± 3.6 on day 12 and 102 ± 4.8 on day 25. That in the control group was 0.36 ± 0.12 on day 4, 9.6 ± 2.4 on day 12 and 70.8 ± 3.6 on day 25. The amount of CFU-GM in MSC-treated groups was significantly different compared with the control level at

Figure 1. The *sry* gene of mesenchymal stem cells (MSCs) of mice was detected on day 30 after irradiation and MSC infusion. BM, bone marrow cells; P, positive control; N, negative control; M, DL-2000 marker; sry, 239 bp.

each observed time point ($p < 0.01$). The percentage of CFU-F in the MSC-treated mice was 45 ± 3.6 on day 4, 36 ± 4.5 on day 12 and 55.8 ± 6.3 on day 25. That in the control group was 16.2 ± 3.6 on day 4, 30.6 ± 5.4 on day 12 and 37.8 ± 3.6 on day 25. The amount of CFU-F in MSC-treated groups was significantly different compared with the control level at each observed time point ($p < 0.01$).

Pathological changes in bone marrow

The bone marrow showed active proliferation in the two groups of mice on day 12 after radiation. The counts of megakaryocites were from 0 to 1 per high powerfield in the control group, and 4–5 in the MSC group. On day 23, all haematopoietic lineages had recovered to the normal ratio. 8–10 megakaryocytes were observed in the control group, whereas 50–60 megakaryocytes were observed in the MSC group (Figure 5).

Cell-cycle and apoptosis

The apoptosis ratio of BMCs was lower in the MSC group than in the control group 24 h after irradiation ($p < 0.05$) (Table 1). The ratio of G_0/G_1 had decreased to a greater level in the MSC group at day 2 ($p < 0.01$). The ratio of G_2/M and S phases was higher in the MSC group ($p < 0.05$) (Table 1).

Survival experiments

Mortality to a level of 100% was induced by 8 Gy TBI within 30 days without transplant of BMCs. The survival of MSCs in the 2.5×10^7 kg^{-1} plus BMCs group (Group D) was 30% after 8 Gy TBI. Survival in the 5×10^7 kg^{-1} plus BMCs group (Group C) was 43%, and in the 1.5×10^8 kg^{-1} plus BMCs group (Group B) was 12%. Finally, the survival of the BMCs alone group (Group A) was only 20% within 30 days. The survival of Groups C and D was significantly different compared with that of Group A. These results are shown in the Kaplan–Meier plots in Figure 6.
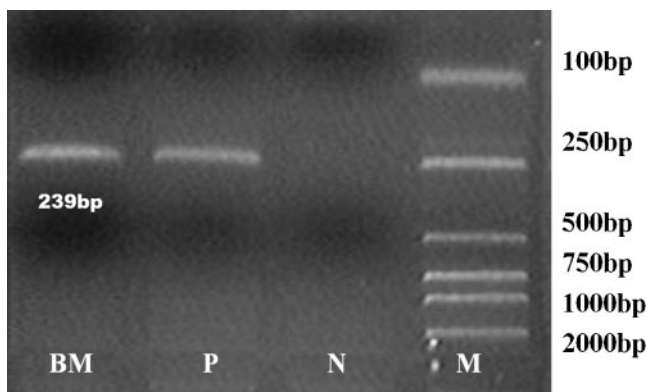
Discussion

In our study, we cultured MSCs from the bone marrow; the MSCs and their capacity for differentiation were identified with monoclonal antibodies. The *sry* gene of MSCs from male donor mice was detected in the bone marrow of recipient mice. The peripheral blood counts showed that the three blood lineages in the MSC groups were higher than those in the control group after 5.5 Gy of irradiation. This knowledge could be beneficial for mice in order to reduce infection and blood complications and, moreover, allow patients to safely overcome the severe stage of ARS. The rapid recovery of thrombocytes in the MSC groups implied that MSCs dramatically enhance the recovery of haematopoiesis because platelets increase very slowly after irradiation injury.
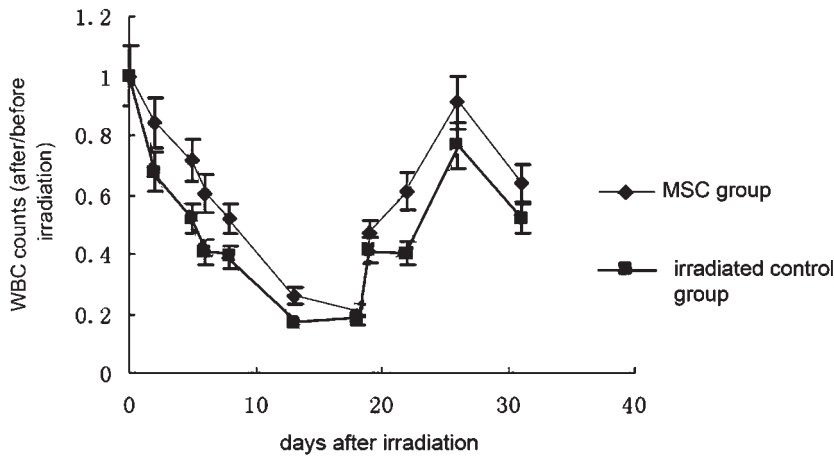
Figure 2. Effect of mesenchymal stem cells (MSCs) on the leukocyte count in sublethally irradiated mice. Each data point represents the mean $\pm$ standard deviation obtained from eight mice. Statistical significance was determined by comparison between the irradiated control group and the MSC group ($p < 0.05$) (every group, $n = 20$). WBC, white blood cell.
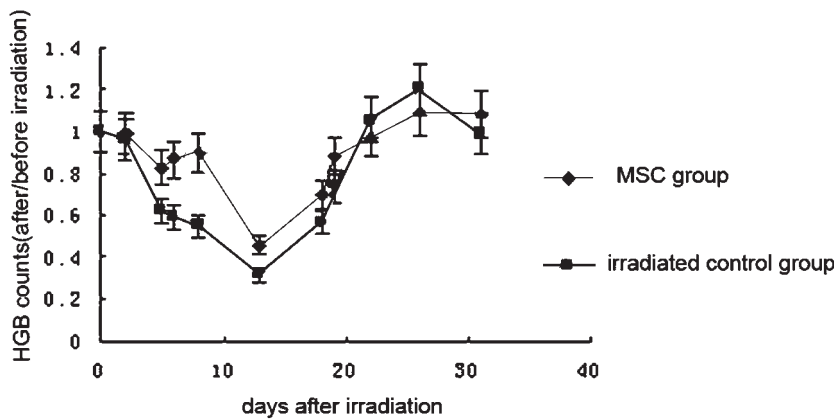

Figure 3. Effect of mesenchymal stem cells (MSCs) on the haemoglobin (HGB) count in sublethally irradiated mice. Each data point represents the mean $\pm$ standard deviation obtained from eight mice. Statistical significance was determined by comparison between the irradiated control group and the MSC group on days 5, 6, 8, 13, 18 and 19 ($p < 0.05$).
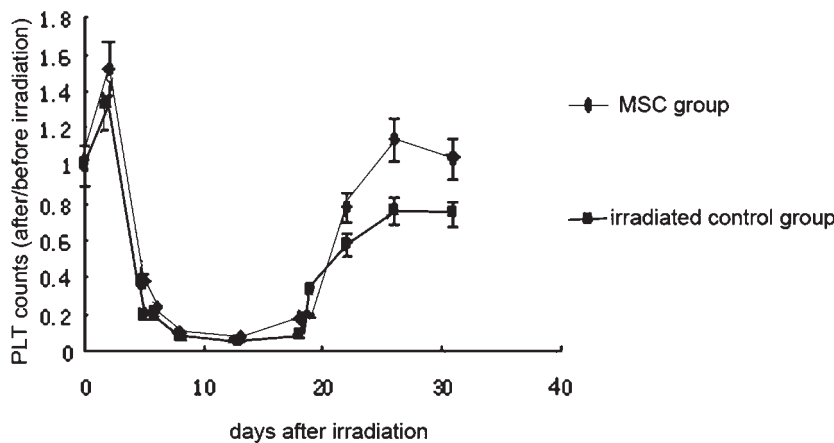

Figure 4. Effect of mesenchymal stem cells (MSCs) on the platelet (PLT) count in sublethally irradiated mice. Each data point represents the mean $\pm$ standard deviation obtained from eight mice. Statistical significance was determined by comparison between the irradiated control group and the MSC group on days 20, 26 and 31 ($p < 0.05$), (every group, $n = 20$).

Table 1. The cell-cycle and apoptosis of bone marrow cells in two irradiated groups (%)

Time post irradiation		G0–G1	S	G2–M	Apoptosis
	Normal	70.81 ± 3.44	23.6 ± 3.76	5.45 ± 0.82	0.68 ± 0.22
	Control	76.77 ± 4.21	7.81 ± 1.56	14.42 ± 3.14	2.94 ± 0.43
6	MSCs	77.75 ± 3.32	10.37 ± 2.45	11.88 ± 3.42	2.36 ± 0.16
	Control	79.84 ± 3.28	3.81 ± 3.73	16.35 ± 2.17	5.24 ± 0.24
12	MSCs	75.33 ± 4.12	3.07 ± 1.93	21.6 ± 1.98	6.36 ± 0.34
	control	82.32 ± 3.54	4.27 ± 3.72	13.41 ± 2.78	4.48 ± 0.33
24	MSCs	86.14 ± 4.45	4.9 ± 1.11	8.96 ± 1.56	2.59 ± 0.45^a
	Control	64.83 ± 3.98	32.68 ± 3.45	2.49 ± 1.23	1.4 ± 0.45
72	MSCs	57.69 ± 2.05^a	38.31 ± 4.22^a	4.23 ± 0.87	0.8 ± 0.32

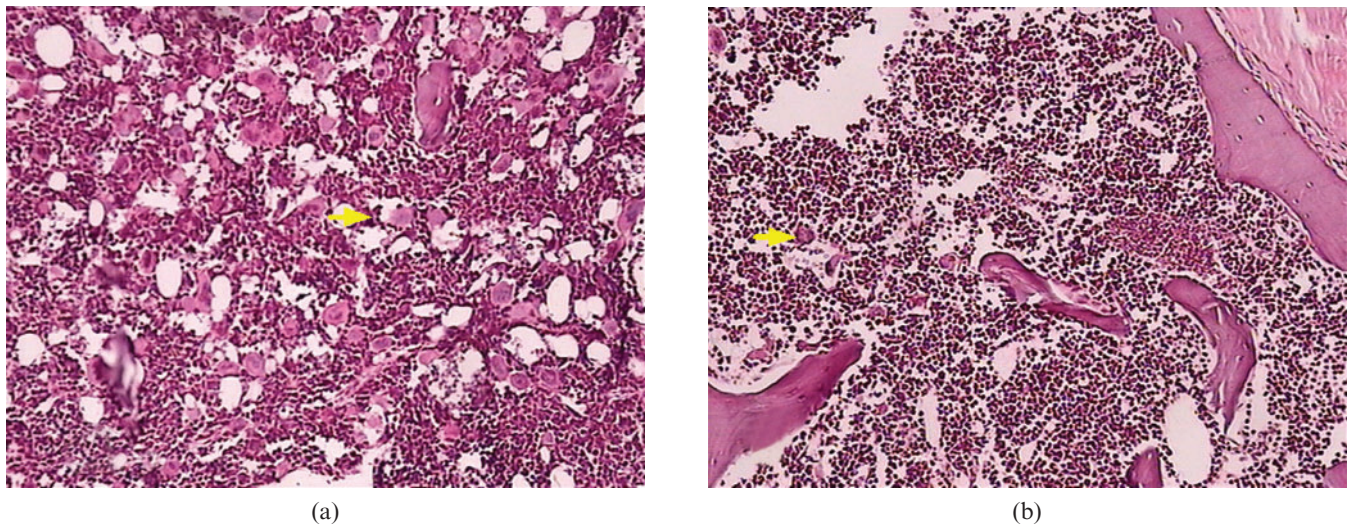$^a p < 0.05$ compared with the control in each time point (every group, $n = 20$). MSCs, mesenchymal stem cells.

Figure 5. The pathological section of the sternum medulla in the (a) mesenchymal stem cell and (b) control groups (haematoxylin and eosin, ×200).The arrows indicate megacaryocytes (every group, $n = 5$).

Counts of the CFU-GM showed that the granulocyte macrophage line had greater radiosensitivity and faster depression and recovery. The CFU-GM counts were higher in the MSC groups than in the control group. MSCs may alleviate the progenitor's injuries by ameliorating the microenvironment and secreted cytokines; they may also improve proliferation. The stromal cells are the primary cells damaged by radiation in the microenvironment [21]. The CFU-F counts in the control group decreased slowly and lingered at a low level for a long time. They also recovered slowly, with levels still <50% of the normal counts at day 25. The counts in the MSC groups had recovered to >60% of the normal counts at day 25, showing that the MSCs protected and improved the microenvironment.

The cell-cycle is a complicated process co-regulated by a series of proteins, kinases and cytokines. Irradiation can cause cell-cycle disorder in the bone marrow, including G_1 and G_2/M blocking. DNA synthesis was depressed after irradiation >2 Gy [22, 23]. Our data showed that MSCs enhanced progression of cells from the resting phase to the cell-cycle-accelerated DNA synthesis and mitosis processes, thus progressing the proliferation of haemocytes and taking advantage of haematopoietic recovery. Apoptosis is the most important cause of bone marrow injuries by irradiation in medium- or severe haematopoietic radiation injury [24]. The occurrence of apoptosis is intimately related to oncogene and anti-oncogene regulation. p53 is the important checkpoint protein that controls the switch from G_1 to S in the cell cycle. p53 determines whether cells go into the S phase or undergo apoptosis [25, 26]. The DNA damaged by irradiation activates two kinases: ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related) [27]. These are crucial molecular signals in supervising radiation injury, by ''sensing'' the injury and activating a downstream response component. ATM and ATR phosphorylate the serine of p53 and counteract the depression of Mdm2 to p53. Increased p53 activates the downstream activity of p21, which restricts the dependence of cell-cycle kinases, blocks the S phase and induces apoptosis. Apoptosis of cells in the MSC groups
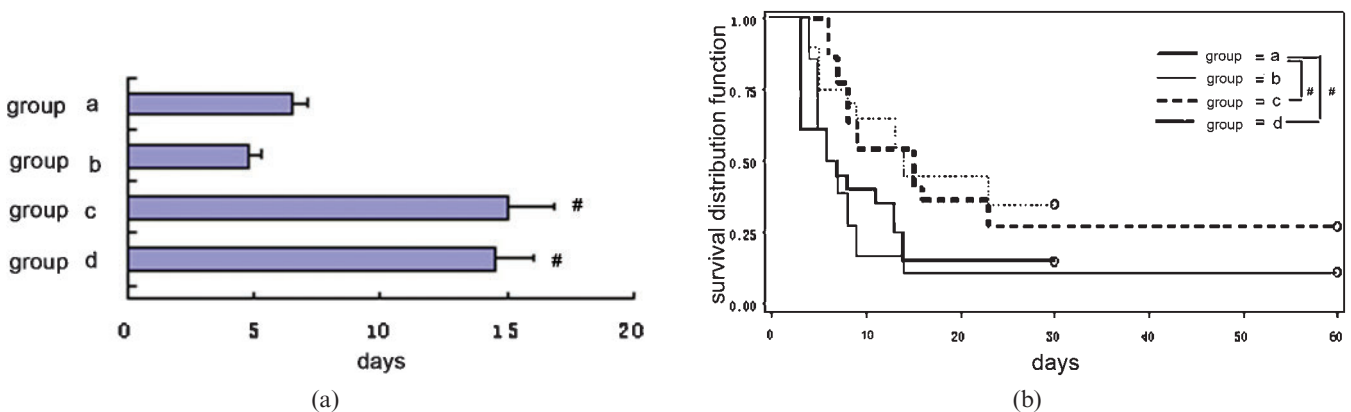

Figure 6. (a) Mean survival time and (b) survival curve for each group. The mean survival time was 8.55 ± 0.94 days in Group A (BALB/c mice treated with 1×10^9 kg^{-1} bone marrow cells (BMCs) after 8 Gy of total body irradiation (TBI)). The mean survival time in Group B (BALB/c mice treated with BMC+1.5×10^8 kg^{-1} MSCs after 8.0 Gy TBI) was 7 days. The mean survival time in Group C (BMCs+5×10^7 kg^{-1} MSCs) was 14.5 ± 1.65 days. The mean survival time in Group D (BMCs+2.5×10^7 kg^{-1} MSCs) was 15.1 ± 1.55 days. # indicates $p < 0.05$ when compared with group A (every group, $n = 20$).

was reduced when compared with that in the control group at 24 h. The data indicate that the cytokines from the MSCs reduced the expression of p53 in irradiated cells. They also caused cells to leave the G_0/G_1 block, accelerated DNA synthesis and mitosis, and decreased apoptosis [28–30].

There are three major mechanisms of MSC-induced haematopoiesis. First, despite a transient increase in cytokines after irradiation, cytokines are comparatively scarce because cell factor receptors are damaged and the tolerance of cytokine receptors is advanced [31, 32]. Therefore, the cytokines from the MSCs can make up for the cytokine shortage. It is known that MSCs secrete a large number of cytokines *in vitro*, including GM-CSF, stem cell factor (SCF), thrombopoietin (TPO), IL-6, IL-11, Flt3 ligand and stromal cell-derived factor-1 (SDF). SCF is the stem cell factor that induced the most primitive of stem cells — granulocytes [33, 34], whereas TPO can stimulate colony-forming megakaryocytes [35, 36]. IL-6 is the multiple-function factor that activates the immune and haematopoietic system, which can activate B cells and improve the number of thrombocytes and granulocytes [37]. IL-11 can enhance the proliferation of all the blood cell lineages [38]. SDF induces transendothelial migration of haematopoietic stem cells to the bone marrow. SDF is classified as a cxc-chemokine and is also a chemoattractant for monocytes and lymphocytes [39]. Second, complete recovery of the haematopoietic micro-environment destroyed by irradiation is complex and occurs slowly [40]. MSCs can differentiate to stroma cells, fibroblast cells and adipose cells, which reconstitute the microenvironment and improve the number of haematopoietic islands. The islands induce stem cell proliferation and differentiation. Third, MSCs protect the haematopoietic cells from apoptosis and enhance the cell-cycle transition after irradiation. One explanation for this may be that MSCs have radioprotective effects by secreting IL-11, SCF, Flt-3L and IL-7 [41].

It is generally believed that the damage induced by high-level irradiation to the haematopoietic system results in the death of mice within a period of 30 days, and mortality can be directly attributed to infections and haemorrhagic complications resulting from neutropenia and thrombopenia. In our study, the high survival rate induced by MSCs was related to accelerated platelets and neutrophilic recovery. It is noteworthy that the survival of the $5 \times 10^7 \, kg^{-1}$ and $2.5 \times 10^7 \, kg^{-1}$ MSC groups showed no statistically significant difference, and that of the $1.5 \times 10^8 \, kg^{-1}$ MSC group was less than that of the BMC group. This finding occurred because MSCs are strongly sticky and favour conglobation. For these reasons, the over dosage of MSCs may be embolise in mice organisation to cause mice death.

MSCs reduced apoptosis, enhanced cell-cycle transition, improved the microenvironment, and accelerated haematopoietic stem cell proliferation and differentiation, and may be an effective treatment strategy for ARS.

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