

Cell and Organ Transplantation. 2022; 10(1): 10-16.
<https://doi.org/10.22494/cot.v10i1.139>

The efficacy of cryopreserved ex vivo expanded rat bone marrow-derived multipotent mesenchymal stromal cells in the repair of radiation injuries in rats



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ABSTRACT

At present, transplantation of multipotent mesenchymal stromal cells (MSCs) as cell therapy for radiation injuries has gained increasing attention since current medical management remains far from satisfactory.

THE PURPOSE of the study is to examine the efficacy of cryopreserved ex vivo expanded bone marrow-derived MSCs (rBM-MSCs) to the repair of radiation injuries on rat models of total and local radiation exposure.

MATERIALS AND METHODS. The MSCs were derived from bone marrow of non-irradiated female albino rats aged 4 months, short-term ex vivo expanded for two passages and cryopreserved under dimethyl sulfoxide cryoprotection for low temperature storage at -70°C for 6-12 months. The cryopreserved samples from each batch of rBM-MSCs culture were tested for the viability and functional characteristics before being transplanted to rats in experiments in vivo. The acute radiation damages in rats were modeled by total body irradiation (TBI) at doses of 5.5 Gy (TBI 5.5) and 7.0 Gy (TBI 7.0) and locally irradiated in the right hip skin at a dose of 50 Gy. The cryopreserved rBM-MSCs ($1.5 \cdot 10^6$ and $0.5 \cdot 10^6$ cells/animal) were intravenously transplanted within 24 h following TBI and locally injected (twice $1.5 \cdot 10^6$ cells/animals) on days 15 and 21 following thigh irradiation. The efficacy of cryopreserved rBM-MSCs was assessed by survival and hematological study as well as the irradiated skin wound healing assay.

RESULTS. The cryopreserved ex vivo expanded rBM-MSCs were characterized by high level of functional activity with cell viability about 80 %, include at least 8.5 % of the colony forming MSCs and MSCs with ability to adipogenic and osteogenic differentiation. In TBI 5.5 rats, cryopreserved transplanted rBM-MSCs ($1.5 \cdot 10^6$ cells/animal) prevented acute leukopenia in the first critical days of the radiation injury by increasing the number of leukocytes by 3.7 times on day 2 and contributed to a more complete recovery of hematological disorders by increasing the BM cells number and platelet count on day 22, which led to the increase of overall survival up to 100 % with a regain of body weight. In TBI 7.0 rats, the lower transplanted dose of rBM-MSCs ($0.5 \cdot 10^6$ cells/animal) was more effective in terms of general recovery and extended the overall survival time for 6 days. The locally injected rBM-MSCs (twice $1.5 \cdot 10^6$ cells/animals) reduced the severity and promoted the healing of radiation skin wounds according to the results of scoring and wound size assay.

CONCLUSION. The present study confirms that the cryopreserved ex vivo expanded rBM-MSCs were functionally complete for the therapeutic use on rat models of experimental radiation damage and were effective for the recovery of hematopoietic system and severe skin wound after radiation exposure.

KEY WORDS: bone marrow-derived multipotent mesenchymal stromal cells; cell expansion ex vivo; cell cryopreservation; radiation injuries

The radiation injury of normal tissues exposed to ionizing radiation is an important problem of public health. Human exposure to ionizing radiation and the resulting radiation injury have often been associated with

radiation accidents, but it also is the most limiting factor in radiotherapy of oncology patients. In addition, the contemporary world is facing a serious risk usage of nuclear weapons and nuclear terrorism or war that may

result to mass casualties of radiation damages. It is known that radiation damages are more severe in organs and tissues where cells have a high turnover rate, which include skin, hematopoietic system, gut and cerebrovascular system [1]. The current medical management remains far from satisfactory, especially none of proposed countermeasures are ready to be implemented during a mass-casualty scenario. At present, there are only few drugs such as G-CSF (Filgrastim and Pegfilgrastim) and GM-CSF (Sargramostim) which are cytokines manufactured *in vitro* and have been FDA-approved to control hematopoiesis and local inflammatory responses resulting radiation exposure. Unfortunately, they are very costly and shown to be effective for a limited number of conditions [2, 3]. Over the past decade the application of mesenchymal stromal cells (MSCs) as cell therapy for the treatment of radiation injuries has gained increasing attention [4,5]. Although the current clinical use of MSCs is limited to trials for diseases other than radiation injuries, their unique biological features, such as migration, homing, multi-potency and minimal host rejection enable their wider application in medical conditions requiring tissue regeneration [6-10]. MSCs have been originally isolated from bone marrow (BM) and now successfully obtained from different biological sources, including the adult BM-MSCs, adipose tissue, placenta, amniotic fluid, cord blood and fetal tissues [11]. The International Society for Cell & Gene Therapy has established the minimal criteria for defining human multipotent MSCs in order to achieve the uniform characterization of MSCs isolated from different sources [12, 13]. MSC therapy represents an approach that is rapidly gaining acceptance and no other multipotent stem cell therapy has established an extensive safety profile in clinical practice [14]. So far, promising experimental data on the treatment of radiation skin injuries using MSCs have been obtained [15]. The reported advantages of using MSCs as therapeutic agent in cell therapy include the possibility of *ex vivo* concentration and expansion with a differentiation potential determined by the surrounding microenvironment, highly adaptable transplantation by method of the intravenous infusion and rapid distribution to the injury sites because of cell homing properties [16, 17]. One major concern is to stockpile a sufficient number of allogeneic MSCs that would be readily available to provide timely administration in a clinical setting. For this MSCs can be expanded for a relatively short period of time and cryopreserved for point-of-care delivery. Cryopreserved MSCs can be stockpiled and stored for a long time with minimal loss of their potency. Both MSCs banking and testing of the cryopreserved cell quality are necessary before their direct administration. Although human data are normally preferred, the preclinical studies on adequate experimental animal models are an important stage to assess the therapeutic potential, efficacy and safety of MSCs.

The **PURPOSE** of the present study was to examine the efficacy of cryopreserved *ex vivo* expanded bone marrow-derived MSCs in the repair of radiation injuries on rat models of total and local radiation exposure.

MATERIALS AND METHODS

Animals. Female outbred albino rats the age of 4 months (160-180 g) were used for the experiments. The rats were housed under standard conditions in animal rooms at 21 ± 2 °C, 50 ± 10 % of humidity and 12-h light/dark cycle. Commercial rodent diets and water were available *ad libitum*. Only healthy rats were used for the irradiation. All animal studies were carried out in accordance with the international principles of «European Convention for the protection of vertebrate animals used for experimental and other scientific purposes» (Strasbourg, 1998) and norms of biomedical ethics according to the Law of Ukraine «On the Protection of Animal from Cruelty» (Kyiv, 2006) under the control of the Committee for Medical ethics of State Institution «Grigoriev Institute of Medical Radiology and Oncology of National Academy of Medical Sciences of Ukraine».

Isolation and *ex vivo* expansion of rBM-MSCs. Bone marrow aspirate was obtained from femurs of rats ($n = 10$) which were euthanized with an overdose (50 mg/kg) of sodium thiopental (*Brovapharma*, Ukraine).

The cell suspensions were collected by flushing the marrow cavity with 199 Medium (*Biowest*, France) and centrifuged at $300 \times g$ for 10 min. The cell pellets were resuspended in 3 mL of physiological saline (*Yuri Farm*, Ukraine) and the total nucleated bone marrow cells number was counted. Cells were seeded at $4 \cdot 10^5$ nucleated cells/cm² to cultured flask (SPL, Korea) into the growth medium α MEM supplemented with L-glutamine 2 mM (*Biowest*, France), fetal bovine serum 15 % (FBS; *Biowest*, France), streptomycin 50 μ g/mL and penicillin 50 U/mL (*Sigma-Aldrich*, USA) for cultivation under a humidified atmosphere of 5 % CO₂ at 37 °C. After 3 days non-adherent cells were removed and culture medium was changed every 3 or 4 days. The belonging of isolated cells to MSCs was confirmed by the criteria of the cells ability to adhere to plastic, form colonies under standard cultivation conditions and differentiate into adipocytes and osteoblasts in induced culture medium [18]. The adherent cells were passed up reaching 80 % confluence of monolayer and MSCs colonies were identified morphologically. All experiments were performed using cells in their 2 passages. At each passage the number of seeded and harvested rBM-MSCs was counted. Population doubling level (PDL) was determined by formula [11]:

$$PDL = \log N / N_0 \times 3.3$$

N_0 – number of seeded cells;
 N – number of harvested cells.

Cryopreservation of rBM-MSCs *ex vivo* expanded. The cultured rBM MSCs from the 2nd passage were harvested and slowly mixed with α MEM medium supplemented with FBS 15 % and cryoprotectant dimethyl sulfoxide (DMSO) (*neoFroxx GmbH*, Germany) in a final concentration of DMSO 1.4 M. The cell suspensions which included $1-2 \cdot 10^6$ cells/mL were placed into cryotubes (*Nunc*, USA) and put into a container NalgeneMr Frosty Cryo 1 °C Freezing (*Thermo Scientific*, USA). The container was placed in the freezing camera (-70 °C). The cryopreservation process provided a slow cell cooling at a rate of 1 °C/min to $t = -70$ °C and low-temperature storage of rBM-MSCs expanded *ex vivo* lasted for 6-12 months before their use in experiments *in vivo*.

The thawing of the cryopreserved rBM-MSCs suspensions was carried out in a water bath ($+37$ °C). The removal of DMSO was performed by slow dilution of the cryopreserved cell suspensions with Hanks solution (*Biowest*, France) in the volume ratio of 1:5 and centrifugation at $300 \times g$ for 10 min. The cell pellets were resuspended in physiological saline to reach a final concentration for the injections. After cryopreservation the viability and functional characteristics of the *ex vivo* expanded rBM-MSCs were examined.

The cell viability express-test of the cryopreserved rBM-MSCs was carried out with Trypan blue (0.2 %) staining. Cell viability was estimated as the percentage of viable (non-stained) cells to the total cell number in the Goryaev chamber. The colony-forming efficiency (CFE) of the cryopreserved rBM-MSCs was estimated in culture at low seeding dose (10^6 cells/cm²) into 25 cm² culture flask (SPL, Korea) with α MEM supplemented with 20 % FBS with fixed in 96 % ethanol and stained with Giemsa's azur-eosin-methylene blue solution on day 14. MSCs colonies were counted under inverted microscope (*Leica*, Germany). CFE was determined by formula:

$$CFE = nN \times 100 \%$$

n – number of MSCs colonies,
 N – number of the seeded rBM-MSCs.

Adipogenic and osteogenic differentiation of the cryopreserved rBM-MSCs. For adipogenic differentiation rBM-MSCs were seeded at $1 \cdot 10^4$ cells/cm² in 24-well plate (SPL, Korea) in inductive adipogenic differentiation medium: α MEM (*Biowest*, France) supplemented with L-glutamine 2 mM (*Biowest*, France), isobutylmethylxanthine 0,5 mM

(Sigma, USA), insulin 10 µg/mL (Sigma, USA), indomethacin 200 µM (Sigma, USA), dexamethasone 1 µM (Sigma, USA), FBS 10 % (Biowest, France). For osteogenic differentiation rBM-MSCs were seeded at 3·10³ cells/cm² in 24-well plate (SPL, Korea) in inductive osteogenic differentiation medium: αMEM (Biowest, France) supplemented with L-glutamine 2 mM (Biowest, France), ascorbic acid 0.05 mM, beta-glycerophosphate 10 mM (Sigma, USA), dexamethasone 0.1 µM (Sigma, USA), FBS 10 % (Biowest, France). The media were changed twice a week. The cultivation was carried out for 3 weeks. Then the cultures were carefully rinsed with Hanks solution and fixed in Ca-formol for 30 min (+4 °C) and stained. Adipogenic cells were detected by intracellular accumulation of neutral lipids after staining with Oil Red O. Differentiating osteogenic cells were detected after staining with Fast Blue RR Salt (Sigma, USA) for alkaline phosphatase expression.

THE RAT MODELS OF RADIATION INJURY

Total body irradiation. The rat model of acute radiation injury was carried out by bilateral total body irradiation (TBI) on a CLINAC (6 MeV) linear accelerator (Varian Medical System, Inc., USA) at a sublethal dose 5.5 Gy (TBI 5.5) and absolute minimum lethal dose 7.0 Gy (TBI 7.0). The rats were irradiated in a special 15×15 cm plastic box with holes for ventilation at room temperature. TBI were delivered through 5-mm plastic for homogeneous dose distribution. The dose rate in the center of the plastic box was 0.76 Gy/min and the dose delivery was calibrated with a UNIDOS universal dosimeter along with an ionization chamber TW 30001-2127 by physicist-dosimetrists. In a survival study the irradiated rats were randomly divided into control TBI 5.5 (n = 20), TBI 7.0 (n = 20) rats and rats treated with transplanted cryopreserved rBM-MSCs (n = 55). The cryopreserved allogeneic rBM-MSCs at a conventional dose (1.5·10⁶ cells/animal) were administered into the tail vein within 24 h following the TBI 5.5 (n = 20) and TBI 7.0 (n = 20). In additional study with TBI 7.0 (n = 15), a lower dose of transplanted rBM-MSCs (0.5·10⁶ cells/animal) was also used. The control TBI rats were injected with the 1 mL of physiological saline as vehicle at the same time points. The overall survival rate and mean survival time with median (Me) within 30 days were evaluated. The clinical symptoms of radiation damage (the weight loss, mobility, body posture, diarrhea, facial oedema) were monitored in each rat daily up to 30 days. At the end of the experiment the rats were euthanized with an overdose (50 mg/kg) of sodium thiopental (Brovapharma, Ukraine). In hematological study, TBI 5.5 rats were used only. The hematological parameters were monitored on days 0, 2, 15 and 22 on surviving rats both in control TBI 5.5 and TBI 5.5 with transplanted rBM-MSCs groups. The peripheral blood samples (20 µL) were collected from the tail vein of rats (n = 5-8 on each time point in both groups) into 1 µL of 0.5 M EDTA (Sigma, USA) coated blood collection tubes (Sarstedt, Newton, USA) to automatically count leukocytes, platelets and erythrocytes on a hematology analyzer RT-7600 (Rayto, China). In addition to studying with TBI 5.5 (n = 45), the BM cells number was counted. For BM cell number analyses, the rats were euthanized with an overdose (50 mg/kg) of sodium thiopental (Brovapharma, Ukraine) on days 0, 2, 15 and 22 after TBI 5.5. The BM cells were obtained from femurs of euthanised rats (n = 5 on each time point in both groups) by the method described above. BM cells number counting was carried out in a Goryaev chamber by the standard method with acetic acid 3 %. The native control rats (n = 25) were sham irradiated and used in hematological study as well.

Local irradiation. The rats (mean mass 170 g) were anesthetized by intraperitoneal injection of sodium thiopental 12.5 mg/kg (Brovapharma, Ukraine) and aminazin 12.5 mg/kg (Arterium Corp., Ukraine) and the right hip skin was locally irradiated at a single dose of 50 Gy (dose rate 725 cGy/min) on a MultiRad 225 (Faxitron Bioptics LLC, USA). Hair from the dorsal surface of the rat legs was removed 24 h before local irradiation. The irradiation area for each rat was 25×25 mm², the distance «source - object» 51 cm and the Cu filter 0.3 mm was used. The rats were randomly divided into control irradiated (n = 20) and treated with injected cryopreserved rBM MSCs (n = 25). In the experiment we used the twice

injection of rBM-MSCs at identical doses 1.5·10⁶ cells/animal on days 15 and 21 within manifestations of acute radiation reactions consistent with [19]. For rBM-MSCs delivery, 1 mL of cells suspension (1·10⁶ cells/mL in saline) was injected with 1 mL syringe subcutaneously at each site around the entire skin wound. As a control, 1 mL PBS was injected into each rat in the control group.

The irradiated skin wound score and wound healing assay. The radiation damaged skin was assessed by visual scoring of the acute skin reaction estimated every three days until 8 weeks after irradiation and data were presented as means. For scoring, the modified an in-house skin score system was used as follows: 0 – the skin shows no change; 0.5 – slight erythema; 1.0 – mild erythema; 1.5 – true porphyritic erythema; 2.0 – dry desquamation and reduced sweat; 2.5 – dry desquamation in more than 50 % of the area; 3.0 – moist desquamation in a small area and moderate edema; 3.5 – the confluent moist desquamation in most of the area, pitting edema and brown crusts; 4.0 – the bleeding erosion, ulceration and necrosis. Our scoring scale for preclinical study on rat model was based on recommendations of the International Union Against Cancer for clinical assessment the severity of skin radiation injury to more closely align with scoring used clinically. Unlike the Kumar scale [20], our scoring system has a more compressed scale with only four-point-range scoring scale (0–4) and does not include open wound, full thickness skin loss as a score of 5 point. For wound size analysis, the irradiated area of each rat was photographed with a digital camera at each time point. From these images, the percentage of the wounded area was determined by calculating the ratio of the ulcerated area to the total irradiated area using ImageJ, version 1.47 (NIH, Bethesda, MD, USA). The ulcerated area was defined as scabbing, crusting, or desquamation. The irradiated area was defined as the area where hair loss was observed. The wound healing for each rat was assayed and data was averaged.

Statistical analysis. For survival data the Kaplan-Meier survival analysis was used and the difference between the survival curves was estimated by log rank test. The other data were compared and evaluated by unpaired Student's t-test and nonparametric Mann-Whitney U-test using SigmaStat 5.0 statistical software (Systat Software Inc., USA). All the data were presented as mean and standard deviation (M ± m). The difference between the studied parameters was considered statistically significant at a value of p < 0.05.

RESULTS AND DISCUSSION

Characterization of the cryopreserved ex vivo expanded rBM-MSCs

It was determined that rBM-MSCs approximately doubled at each cultivation passage according to the calculation of PDL as (1.79 ± 0.26) at passage 1 and as (1.64 ± 0.29) at passage 2. The cryopreserved samples from each batch of cultured rBM-MSCs were characterized by a high level of cell viability (81.7 ± 1.3) % and CFE (8.5 ± 1.4) %. Thus, after low-temperature storage (-70 °C) for 6-12 months cryopreserved rBM-MSCs at a low seeded dose of 10⁶ cells/cm² formed discrete colonies of various sizes on day 14 of cultivation (Fig. 1 A) and kept the ability to be induced to adipogenic (Fig. 1 B) and osteogenic (Fig. 1 C) differentiation. These results confirmed that cryopreservation conditions ensured the high viability and functional activity of extended rBM-MSCs intended for therapeutic administration to rats with radiation damage in the experiments *in vivo*.

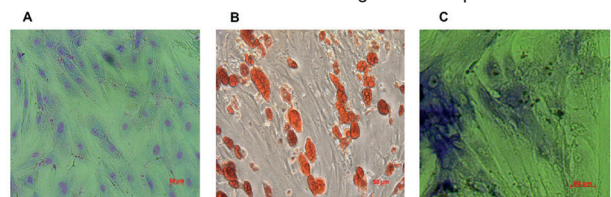


Fig. 1. Micrographs of colony (A) and differentiating adipogenic (B) and osteogenic (C) cells derived from cryopreserved rBM-MSCs (passage 2). The cultures were stained with (A). Giemsa's azur-eosin-methylene blue solution; (B) with Oil Red O on lipid accumulations in vacuoles (red) and (C) with Fast Blue RR salt on expression of alkaline phosphatase activity (dark). Scale bar = 50 µm

SURVIVAL ANALYSIS

In survival study the mortality of TBI 5.5 rats was observed within 7-14 days and TBI 7.0 rats within 3-20 days that corresponded with 20 % (LD20/30) and TBI 7.0 with 67 % (LD67/30) over the course of 30 days. The mean survival time of TBI 5.5 rats was (11.0 ± 1.2 , Me = 10.5) days and TBI 7.0 lowered to (8.7 ± 0.9 , Me = 7.0) days, respectively. In TBI 7.0 rats a significant drop in body weight and diarrhea coincided with the increased lethality between days 5 and 14. As shown in the **Fig. 2 A, B** the transplanted cryopreserved rBM-MSCs improved the survival of the lethally irradiated rats, but the effect was determined by the dose of radiation exposure and the cell doses of transplanted rBM-MSCs. The greatest therapeutic effect was observed in TBI 5.5 rats with the rBM-MSCs at a transplanted dose $1.5 \cdot 10^6$ cells/animal. The overall survival rate was increased to 100 % and all of the irradiated rats survived for up to 30 days (**Fig. 2 A**). In our study no mortality of the TBI 7.0 rats with the transplanted rBM-MSCs were recorded in the critical period within the first 7 days of acute radiation injury development. In this time point the survival effect was observed with the both cell doses of the transplanted rBM-MSCs (**Fig. 2 B**). In the follow-up observation (up to the 30th day of the experiment) the rBM-MSCs at the lower transplanted dose ($0.5 \cdot 10^6$ cells/animal) were more effective than at the higher dose ($1.5 \cdot 10^6$ cells/animal) in terms of survival rate and general recovery of the irradiated rats (**Fig. 2 B**). Nonetheless, the mean survival time in TBI 7.0 rats with the both transplanted doses of rBM-MSCs was extended by around 6 days to (13.0 ± 1.1 , Me = 12.5) days and (14.0 ± 1.0 , Me = 13.0) days, respectively.

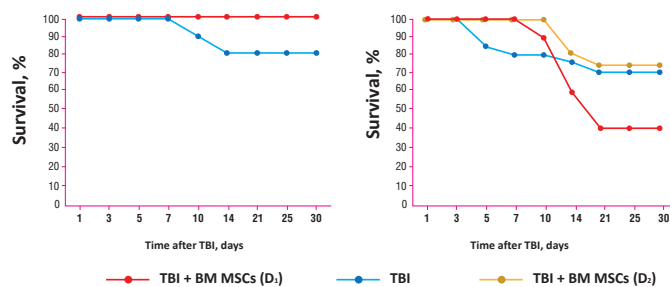


Fig. 2. The survival rate of TBI rats with cryopreserved rBM-MSCs transplantation. (A) TBI 5.5 and cryopreserved rBM-MSCs ($1.5 \cdot 10^6$ cells/animal); (B) TBI 7.0 and cryopreserved rBM-MSCs ($1.5 \cdot 10^6$ cells/animal) (D1) and ($0.5 \cdot 10^6$ cells/animal) (D2).

HEMATOLOGICAL ASSAY

Based on survival data the hematological parameters were monitored only in the TBI 5.5 rats because all of the rats with transplanted rBM-MSCs survived under this dose of radiation exposure and it was possible to evaluate the hematology recovery over a 30-day period. It is generally known that the radiation damage of the hematopoietic system is the principal cause of mortality and could be directly associated with lethality in radiation exposure doses from 1 to 10 Gy [1]. In our study the severe hematological disorders in control TBI 5.5 rats were observed. As shown in the **Fig. 3 A**, the leukocyte number at the acute period on day 2 dropped rapidly by 4.1 times ($p = 0.001$) and then on day 22 decreased by 3.2 times ($p = 0.001$) compared to non-irradiated level (**Fig. 3 A**). Platelet numbers dropped slower by 1.8 times ($p = 0.027$) on day 8 and did not recover completely at the endpoint of observation (**Fig. 3 B**). Moreover, a primary BM depletion was detected. The total BM cells number was dramatically decreased by 4.3 times ($p = 0.005$) on day 2 and then by 7.4 times ($p = 0.001$) on day 4 (**Fig. 3 C**). Compared to the irradiated control, early treatment by cryopreserved rBM-MSCs at a dose of $1.5 \cdot 10^6$ cells/animal within 24 h after exposure prevented acute leukopenia in the TBI 5.5 rats over the crucial first few days in the radiation damage development and enhanced the total BM cells number as well as the platelets level at the recovery period. In rats with transplanted rBM-MSCs the leukocytes number on day 2 was 3.7 times higher ($p = 0.003$) and platelet num-

ber on day 22 increased 2.1 times ($p = 0.001$) relative to TBI 5.5 control (**Fig. 3 A, B**). Respectively a complete recovery of BM cell number was found starting from day 15 (**Fig. 3 C**). Thus, these results suggested that the transplanted rBM-MSCs contributed to a more rapid and sustained recovery of hematological parameters with increasing the overall survival time to respond the exposure.

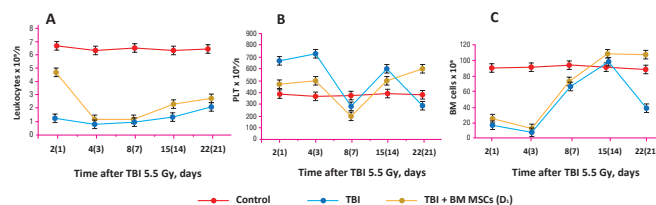


Fig. 3. The leukocytes number (A), the platelet number (B) in the peripheral and the BM cells number (C) in TBI 5.5 rats with cryopreserved rBM-MSCs transplantation ($1.5 \cdot 10^6$ cells/animal). Each data point represents the mean \pm SEM ($n = 5-8$), $p < 0.05$ compared to control TBI 5.5.

At present, rBM-MSCs have been studied more extensively for therapeutic use in hope of understanding the mechanisms of their recovery activity [21]. It was proved that transplanted rBM-MSCs protect the hematopoietic cells from apoptosis and induce proliferation and differentiation of stem and progenitor hematopoietic cells reduced after irradiation [22]. Complete recovery of the hematopoietic microenvironment destroyed by irradiation is complex and occurs slowly. It was demonstrated that transplanted rBM-MSCs reconstitute the hematopoietic microenvironment in BM without significant engraftment into BM. Both *in vitro* and *in vivo* (including clinical) data indicate that the addition of MSC therapy to standard care can promote more rapid recovery of the hematopoietic system. MSCs achieve their therapeutic effects by constitutively secreting a broad range of hematopoietic cytokines, such as IL-6, IL-7, IL-8, IL-11, IL-14, IL-15, macrophage colony-stimulating factor, Flt-3 ligand, and stem-cell factor, IL-1 α induces MSC production of IL-1 α , leukemia-inhibiting factor, G-CSF, and GM-CSF. *In vitro* evaluations have provided detailed evidence that these factors, as well as other members of the MSC secretome (the array of proteins produced and secreted by MSCs), induced the growth of hematopoietic stem cells (HSCs) and their progenitors in an *ex vivo* environment [23, 24]. It is generally believed that the radiation damage results in the lethality of rats within a period of 30 days that can be directly attributed to the hematopoietic system complications. In our study, the 100 % survival rate of sublethally irradiated rats with cryopreserved rBM-MSCs at transplanted dose to $1.5 \cdot 10^6$ cells/animal was related to preventing leukopenia and accelerated platelet and BM cell number recovery. Nonetheless, in the survival rate of rats irradiated by the high lethal dose with cryopreserved rBM-MSCs transplanted at a dose of $0.5 \cdot 10^6$ cells/animal showed no statistically significant difference relative to irradiated control. The increasing of transplanted dose $1.5 \cdot 10^6$ cells/animal led to the worsening of the survival results. This finding is consistent with the data from studies on animals by other authors [25, 26]. Taken together, data suggest that the therapeutic efficacy of BM-MSCs is limited at high lethal doses of whole body irradiation. One explanation for this may be that MSCs have immunomodulating activity [27, 28]. For these reasons, the over dosage of BM-MSCs can enhance the intense immunosuppression that is typically characterized by the manifestation of acute radiation injury by a high lethal dose of irradiation [29].

RADIATION SKIN WOUND

The cryopreserved rBM-MSCs expanded *ex vivo* were also examined for the treatment of acute radiation skin wounds following local irradiation. In general, the human skin response to ionizing radiation is highly complex and dependent on the conditions of the exposure. Early skin radiation effects are characterized by the damage to epidermis while late effects arise from the injury to the dermal vasculature [30]. Acute skin radiation reactions begin within hours as a transient erythema which

subsides after 1 to 2 days and then a more intense erythematous reaction follows. The dry and moist desquamation is typically a reaction manifest of acute radiation skin damage within 2 to 6 weeks, which may occur with a secondary ulceration at 6 weeks or longer. Between 8 and 16 weeks the dermal ischemia and dermal necrosis may develop [19]. In our model the first observable changes true erythema (hyperemia of 1.5) and hair loss were observed in control irradiated rats on day 7. Then the dry desquamation with peeling and appearance of yellow crusts (scores 2.0 – 2.5 units) developed. By day 14 the depilation, swelling, mild erosion and slight seepage were presented. The peak of radiation wound manifestation was observed from day 28. The moist desquamation was indicated throughout the entire skin layer of the irradiated area in all of control rats and skin scores ranged from 3 to 3.5 (Fig. 4 A, B).

The irradiated skin was erosive with red edema, moist areas of seepage, which further formed ulcers in the individual rats. The severe radiation ulceration with necrotic changes (scores 4.0) were exhibited in 22.2 % rats in the irradiated control. At the endpoint of study the wounded skin began to heal gradually, but the healing was slow and still incomplete after 8 weeks (Fig. 4 C, D). The twice local injection of cryopreserved rBM-MSCs at a dose $1.5 \cdot 10^6$ cells/animal on days 15 and 21 reduced the manifestation of acute reactions and severity of the radiation skin wound and promoted healing of the irradiated skin. On day 28 the wound scores decreased to 2.5-3.0 and the skin was presented by the appearance of yellow crusts without pronounced inflammation. The scabs in the irradiated area were thinner, lighter and superficial (Fig. 4 E, F). At the follow-up period the irradiated skin had exudation and erosion, but the depth and area of the wounded skin was significantly milder in comparison with that in the control irradiated rats. In the rats with injected rBM-MSCs, the skin reactions were limited by only moist desquamation without evolving to ulceration and displayed an important healing. On day 42 weeks after the irradiation new epithelial growth was observed at the damage site (Fig. 4 G, H).

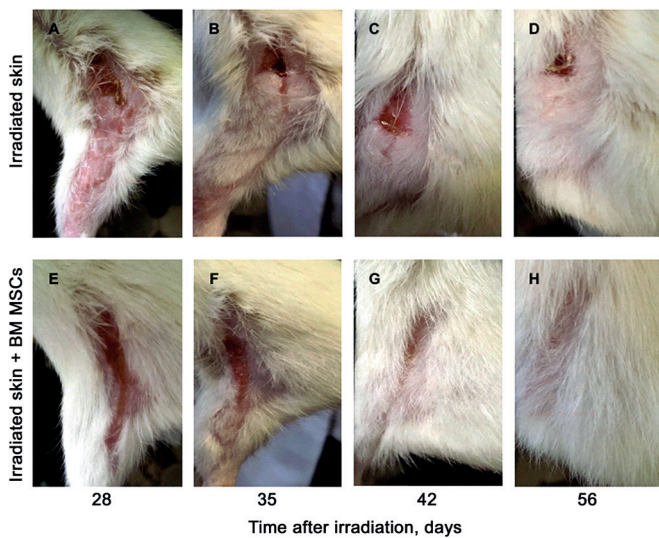


Fig. 4. The photo of rats skin wounds following local irradiation (50 Gy) only (A-D) and local irradiation with cryopreserved rBM-MSC injections (E-H).

The wound scores significantly decreased (Fig. 5 A) as well as the wound sizes on day 28 were 1.5 times smaller ($p = 0.034$) and on day 42 – 1.9 times ($p = 0.013$) than those in the control irradiated rats (Fig. 5 B). Thus, it was confirmed that the locally injected cryopreserved rBM-MSCs led to accelerated and more complete healing of radiation-induced skin injury. The efficacy of MSC treatment in wound healing has been shown in preclinical study on different models, including radiation exposure [31]. It is reported that BM-MSCs differentiate into the cell type that has been damaged and thus promote the repair of wounds [32]. François et al. found that human BM-MSCs injected into irradiated mice migrate towards the damaged skin [33]. However, another study demonstrated that the paracrine mechanisms such as the production of cytokines and proangiogenic factors have contributed to MSC promotion of radiation skin wound healing [34-36].

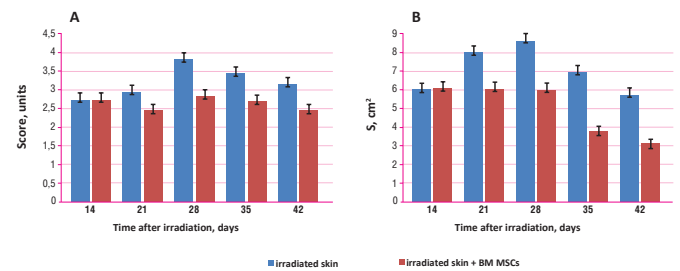


Fig. 5. The radiation wound score and wound healing assay in skin following local irradiation 50 Gy (A) and local irradiation with cryopreserved rBM-MSCs injections (B).

To sum up, the present study confirm that the cryopreserved rBM-MSCs ($1.5 \cdot 10^6$ cells/animal) demonstrated the beneficial effect on the recovery of hematopoietic system and overall survival at a sublethal dose 5.5 Gy of whole body irradiation as well as the severe radiation skin wound at their twice injection after 50 Gy local irradiation. However, to fully explain the therapeutic potential of cryopreserved ex vivo expanded rBM-MSCs the further studies using different radiation doses, time points, and BM-MSCs dosage are needed.

CONCLUSION

1. **Cryopreserved ex vivo expanded rat BM-MSCs had functionally complete cell viability of 80 %, CFE of 8.5 % and potential to adipogenic/osteogenic differentiation. It is indicated by the feasibility of their therapeutic use in preclinical studies on rat models.**
2. **Cryopreserved ex vivo expanded rBM-MSCs demonstrated the therapeutic effect at transplanted dose of $1.5 \cdot 10^6$ cells/animal in rats with a sublethal dose 5.5 Gy of whole body irradiation by preventing leukopenia and accelerated platelets and bone marrow cells number recovery resulted in an increase of the overall survival up to 100 %. In rats with absolute minimum lethal dose 7.0 Gy the therapeutic efficacy of rBM-MSCs at a transplanted dose $1.5 \cdot 10^6$ and $0.5 \cdot 10^6$ cells/animal was limited.**
3. **The twice injection of cryopreserved rBM-MSCs ($1.5 \cdot 10^6$ cells/animal) on days 15 and 21 after 50 Gy of local irradiation reduced the severity and promoted the healing of radiation skin wounds.**

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The authors have no conflicts of interests to declare with acknowledgement of technical assistance in providing the experiments.

УДК 614.876+616.419

Ефективність кріоконсервованих після експансії *ex vivo* мультипотентних мезенхімальних стромальних клітин з кісткового мозку щура при відновленні радіаційних уражень у щурів



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РЕЗЮМЕ

В даний час застосування мультипотентних мезенхімальних стромальних клітин (МСК) як клітинної терапії радіаційних ушкоджень привертає все більшу увагу, тому що поточні можливості лікування даної патології залишаються далекими від задовільних.

МЕТА ДОСЛІДЖЕННЯ: полягала у вивченні ефективності кріоконсервованих після експансії *ex vivo* МСК, отриманих з кісткового мозку щурів, для відновлення радіаційних уражень на моделях тотального та локального опромінення у щурів.

МАТЕРІАЛИ ТА МЕТОДИ: МСК були отримані з кісткового мозку (КМ-МСК) неопромінених самок білих щурів віком 4 місяці, короткочасно культивовані протягом двох пасажів і кріоконсервовані під захистом диметилсульфоксиду для низькотемпературного зберігання при -70°C протягом 6-12 місяців. Кріоконсервовані зразки з кожної партії культури КМ-МСК перевіряли на життєздатність та функціональну активність перед трансплантацією щурам в експериментах *in vivo*. Гострі радіаційні ураження у щурів моделювали шляхом тотального опромінення всього тіла у дозах 5,5 Гр і 7,0 Гр та локального опромінення шкіри правого стегна у дозі 50 Гр. Кріоконсервовані КМ-МСК у дозі $1,5 \cdot 10^6$ і $0,5 \cdot 10^6$ клітин/тварину трансплантували внутрішньовенно через 24 години після тотального опромінення та вводили місцево (двічі $1,5 \cdot 10^6$ клітин/тварину) на 15-й і 21-й день після локального опромінення. Оцінку ефективності кріоконсервованих КМ-МСК проводили за показниками летальності та гематологічних досліджень, а також аналізу загоєння опромінених ділянок шкіри у щурів.

РЕЗУЛЬТАТИ. Кріоконсервовані після експансії *ex vivo* КМ-МСК характеризувалися високим рівнем функціональної активності з життєздатністю клітин близько 80 %, з колонієутворюючою ефективністю 8,5 % та зі здатністю до адипогенного і остеогенного диференціювання. У щурів, опромінених у дозі 5,5 Гр, трансплантовані кріоконсервовані КМ-МСК у дозі $1,5 \cdot 10^6$ клітин/тварину попереджали гостру лейкопенію в перші критичні дні радіаційного ураження шляхом збільшення на 2 добу кількості лейкоцитів у 3,7 рази та на 22 добу сприяли більш повному відновленню кількості клітин кісткового мозку і тромбоцитів, що призводило до зростання загальної виживаності до 100 % із відновленням маси тіла тварин. У щурів, опромінених у дозі 7,0 Гр, менша трансплантована доза КМ-МСК $0,5 \cdot 10^6$ клітин/тварину була більш ефективною за зниженням летальності та подовженням середньої тривалості життя на 6 днів. Місцеве введення КМ-МСК двічі по $1,5 \cdot 10^6$ клітин/тварину зменшувало тяжкість радіаційного ураження шкіри та прискорювало загоєння опромінених ділянок шкіри у щурів.

ВИСНОВКИ. Це дослідження підтверджує, що кріоконсервовані після експансії *ex vivo* КМ-МСК були функціонально повноцінними для терапевтичного використання на експериментальних моделях радіаційних уражень у щурів та були ефективними для відновлення кровотворної системи та опромінених ділянок шкіри після тотального і локального опромінення.

КЛЮЧОВІ СЛОВА: мультипотентні мезенхімальні стромальні клітини кісткового мозку; експансія клітин *ex vivo*; кріоконсервація клітин; радіаційні ураження