International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 12 (3), pp. 001-008, March, 2018. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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Full Length Research Paper

Effect of 60Co radiation on mesenchymal stem cells (MSCs) proliferation and differentiation

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Accepted 20 January, 2018

Mesenchymal stem cells (MSCs) are undifferentiated multipotent cells which reside in various human tissues and have the potential to differentiate into osteoblasts, chondrocytes, adipocytes, fibroblasts and other tissues of mesenchymal origin. In this study, we investigated the effect of 60Co radiation on the proliferation and differentiation of MSCs. MSCs were treated with increasing radiation doses to assess the effect on MSC. Results showed that 2 Gy of 60Co radiation did not significantly affect MSCs. When compared with the control group and 2Gy of 60Co radiation group, the MSCs viability after 4 Gy of 60Co radiation markedly decreased ($p < 0.05$). Two weeks of 8 and 12 Gy of 60Co radiation induced all cell death. After the 5-Aza treatment, the expression of myocardial- specific protein, C-TNT and -MHC was not detected in the MSCs which were pretreated by 60Co radiation. In conclusion, middle dose (4 Gy) of irradiation induces MSCs morphological changes, as well as alteration in both proliferation and differentiation potentials of MSCs. 5- Aza treatment did not induce both proliferation and differentiation of MSCs because high dose (8 and 12 Gy) of irradiation killed all cells.

Key words: Mesenchymal stem cells, radiation, proliferation, differentiation.

INTRODUCTION

Mesenchymal stem cells (MSCs) are adult stem cells traditionally found in the bone marrow. However, mesenchymal stem cells can also be isolated from other tissues including cord blood, peripheral blood, fallopian tube, and fetal liver and lung. Multipotent stem cells, MSCs differentiate to form adipocytes, cartilage, bone, tendons, muscle and skin. Mesenchymal stem cells are a distinct entity to the mesenchyme, embryonic connective tissue which is derived from the mesoderm and differentiates to form hematopoietic stem cells (Wilkins et al., 2009; Rubio et al., 2008; Peter et al., 2010).

Mesenchymal stem cells are characterized morphologically by a small cell body with a few cell processes that are long and thin. The cell body contains a large, round nucleus with a prominent nucleolus, which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance. The remainder of the cell body contains a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. The cells, which are long and thin, are widely dispersed and the adjacent extracellular matrix is populated by a few reticular fibrils but is devoid of the other types of collagen fibrils (Wang et al., 2009; Vachiraroj et al., 2009; Yamada et al., 2007).

Mesenchymal stem or multipotent stromal cells (MSCs) are of importance for therapeutic use due to their ability to proliferate, moreover, differentiate into various cell phenotypes. Culturing or collecting the stem cells is usually necessary to obtain the adequate cell numbers for the clinical applications (Ghaedi et al., 2010).

We, therefore, investigated the effect of 60Co radiation on *in vitro* stability, viability and proliferation capacity of MSCs. Additionally, to date, little is known about the effects of 5-aza on MSCs proliferation, growth factors secretion and differentiation. Hence, the present

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Table 1. 60Co radiation.

Radiation dose (Gy)	2	4	8	12
Radiation time (s)	63	138	289	411

study was also designed to determine the effect of 5-aza on MSCs differentiation.

METHODS

Mesenchymal stem cell cultures

Mesenchymal stem cell cultures were prepared in Chinese PLA General Hospital and Postgraduate Medical School (BeiJing, China) according to the method described by Da Silva Meirelles and Nardi (2003); Nardi and Da Silva Meirelles (2006). Cells were dissociated using collagenase type 1 (0.5 mg/mL in DMEM 10 mM HEPES). After washing by centrifugation at 400 × g for 10 min and counting viable cells with trypan blue, the cells were resuspended in DMEM with 10% FBS and 10 mM HEPES in a final concentration of 5 × 10^6 viable cells per ml.

To initiate the cultures, cells were plated in 96-well tissue culture dishes at 3.5 ml/well (1.94 × 10^6 cells/cm^2) and kept in a humidified 5% CO_2 incubator at 37°C for 72 h, when non-adherent cells were removed by changing the medium. Confluent cultures from MSC were incubated with 0.25% trypsin solution containing 0.01% EDTA for detachment and maintained in culture by changing the medium every 3 - 4 days. For the experiments, cells between the 10 and 25th passages were seeded in 96-well plates in DMEM with 10% FBS and 10 mM HEPES in a density of 30,000 cells/well 2 days before the contact with the organotypic culture. Twenty-four hours before the contact, the medium was changed and conditioned during the next 24 h.

60Co radiation and MSCs cells viablity

60Co radiation

MSCs cells (3rd generation) in 96-well plates were exposed to 60Co radiation (dose rate, 165.44cGy/min) at 30°C for 90 s. The irradiated cells were incubated for continuous 4 weeks (Table 1).

Cell shape

After the removal of the culture medium, each well was washed once with Phosphate Buffered Saline (PBS) and the cells attached to the bottom of the plate were fixed and stained with 0.4% crystal violet solution in methanol for 30 min. After the microplate was washed with water and dried, cell shape of each well was observed using the microscope.

Cell viability

MSCs cell growth and viability was measured by adding 0.4% trypan blue in 0.9% saline to a 50% dilution, and cells were counted, using the hemocytometer according to standard procedure (Pienata and Lehr, 1993) . Briefly, 0.5 ml of the trypan blue solution was transferred to a test tube and 0.3 ml of PBS plus 0.2 ml of the trypsinized cell suspension (dilution factor of 5) were added. The final solution was thoroughly and gently mixed and allowed standing for 5 min.

Then a drop of this dye-cell suspension was loaded onto both chambers of the hemocytometer. Cells were examined and counted in duplicates under light microscope at 200 × (Olympus BH2). Concentration and total number of cells were determined, and percentage cell viability was calculated by the formula:

$$\text{Cell iability (\%)} = \{[\text{Number of viable cells (unstained cells)}] \times 100 \text{ Total number of cells}\}$$

MTT assay

Cell viability was checked using MTT assay. The MTT test is a colorimetric assay that measures the cell survival as percentage of cell survival compared to untreated controls (Williams et al., 2002). In brief, 100 l of 0.45 g/l MTT solution was added to wells. Cells were incubated at 37°C for 45 - 60 min to allow colour development and thereafter, 100 l of 20% SDS in DMF:H_2O 1:1 solution was added to the wells. Plates were incubated overnight at 37°C to solubilize the formazan products. Absorbances were measured at the wavelength of 570 nm. The levels of blue colour development in the control wells were designated as 100% viability, and all further comparisons were based on that reference level. Blank values, indicating the absorbance of MTT and 20% SDS in DMF:H_2O 1:1 solutions only, were subtracted from all samples.

Mesenchymal stem cells differentiation

Group and radiation

MSCs in 6-well plates were divided into A and B groups. Group A received no radiation; Group B was exposed to 60Co radiation (dose rate, 165.44cGy/min) for 138s. One day after radiation, culture medium was changed. Then, 10 ml/L 5-aza was added into the two groups (A and B) . The total incubation time with 5-aza was 24 h. After that, culture medium was changed and MSCs were incubated for continuous 28 days.

RNA extraction

The RNA extraction method using guanidine thiocyanate, phenol and chloroform (all from Sigma) was as described (Verhofstedea et al., 1996). RNA extraction using the 'PURESCRIPTR' kit ('body fluid' protocol) was performed according to the instructions of the manufacturer (Gentra Systems, Minneapolis) . Total RNA extracts were used directly for RT-PCR or first stored in small aliquots at -80°C for periods of up to 2.5 years (stock sample) and at -20°C for periods of up to 8 months (working sample). Samples were thawed and re-frozen no more than four times to minimize possible RNA degradation.

Real time-polymerase chain reaction (RT-PCR)

Subsequently, single-stranded cDNA was synthetized from 2.0 g RNA with the use of 5 mol/L random primers (Ambion, Austin, Texas) and 10 U AMV Reverse Transcriptase (Promega, Madison, Wis) in a buffer containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$ (Ambion), 40 U/ L ribonuclease inhibitor (Promega), and 2 mmol/L of dNTP mix (Ambion) in a total volume of 20 L (final concentrations indicated). RT was carried out for 15 min at 25°C and for 90 min at 42°C, and the samples were subsequently heated for 10 min at 92°C as a means of terminating the RT reaction. Having obtained the cDNA, we carried out a PCR reaction using 6 L of the RT reaction mix. To this, 2.5 U Taq

Table 2. Primers used for the analysis of gene expression on RT-PCR.

Gene	Serial number in gene bank	Sequence	Length (bp)
GAPDH		F 5 - CGTATCGGACGCCTGGTT - 3 R 5 - CGTGGGTAGAGTCATACTGGAA - 3	124
C-TNT	M26052	F 5 - CAGCAGCGTATTCGCAATG - 3 R 5 - TTCTTCTTCCGGGCCTCA - 3	119
-MHC	AY701540	F 5 - GTGTACCCTTCAAAGACGC- 3 R 5 - CACCTTGAGGAACCGTCT - 3	202

polymerase (Promega), 0.6 mol/L sense primer, and 0.6 mol/L antisense primer (made by Roche Diagnostics GmbH, Mannheim, Germany) were added. The final reaction volume was 50 L. The tubes were incubated in a Mastercycler personal thermocycler (Eppendorff-Netheler-Hinz GmbH, Hamburg, Germany) at 94°Cfor 2 min to denature the primers and cDNA. The cycling program consisted of 94°C for 90 s, 55 to 57°C for 1 min, 72°C for 2 min (10 min in the last cycle) and comprised 30 to 35 cycles. For each primer set, an increasing number of PCR cycles with otherwise fixed conditions was performed to reveal the optimal number of cycles to be used. This point was determined to be halfway through the exponential phase. Human and rabbit primers that amplified the rabbit sequence for GAPDH, C-TNT, and -MHC are provided in Table 2. PCR products and molecular-weight markers (Boehringer Mannheim GmbH, Mannheim, Germany) were subjected to electrophoresis on 1% agarose gel and visualized by means of ethidium bromide staining. The gels were analyzed by means of laser scanning densitometry with the use of a Gelprinter plus photodocumentation system (TDI, Madrid, Spain) and Zeiss KS-300 software (Imaging System, Münster, Germany).

Statistical analyses

All analyses were performed in duplicate. Data were analyzed by one-way ANOVA using the general linear model procedure of SAS (SAS Inst. Inc., Cary, NC, USA). Differences among means were tested using Duncan's multiple range tests. A significant level of 0.05 was used.

RESULT

Normal MSCs morphology

Most of first-generation MSCs exhibited a spindle shape. A few cells showed a triangular or round or polygonal shape. Third-generation MSCs still showed a spindle shape and displayed better permeability and light refraction.

MSCs morphology after radiation

MSCs morphology was not significantly affected with doses as high as 2 and 4 Gy of 60Co radiation. MSCs morphology started to markedly change with increasing dose of 60Co radiation (Figure 1). Most of the cell death was observed after 8 and 12 Gy of 60Co irradiation. All cells died after 2 weeks of 8 Gy of 60Co irradiation or after 1 weeks of 12 Gy of 60Co irradiation.

Effect of 60Co radiation on MSCs viability

After 2 Gy of 60Co radiation, the MSCs viability as assayed by trypan blue dye exclusive test were above 97%, and no significant difference in MSCs viability was found among the experiment groups ($P > 0.05$) (Figure 2). When compared with the control group and 2 Gy of 60Co radiation group, the MSCs viability after 4 Gy of 60Co radiation markedly decreased ($P < 0.05$). Two weeks of 8 and 12 Gy of 60Co radiation induced all cell death (Figure 3; Tables 3 and 4).

Effect of 60Co radiation on MSCs proliferation

The inhibitory effect induced by the 60Co radiation was related to the dose of radiation treatments, thus to the global radiative energy given to the cells. It should be noted that there was no significant difference ($P > 0.05$) in the number of dead cells between the control and 2 Gy radiation groups. After 4 Gy radiation treatments, the number of viable cells in the treated samples was significantly ($P < 0.05$) reduced with respect to the control sample. After 8 and 12 Gy radiation treatments, all cells were dead (Figure 4; Table 5).

5-aza-induced MSCs differentiation

Four weeks later, the cells were examined for the morphological changes and mRNA expression of myocardial-specific protein, such as C -TNT and -MHC. In the group A, the MSCs cells showed various morphologies such as a long fusiform shape (Figure 5A). However, MSCs cells cultured in group B (Figure 5B) showed a spindle shape and cobblestone-like shape. Complete cell fusion and myotube formation were not detected in group A and B after 4 weeks culture.

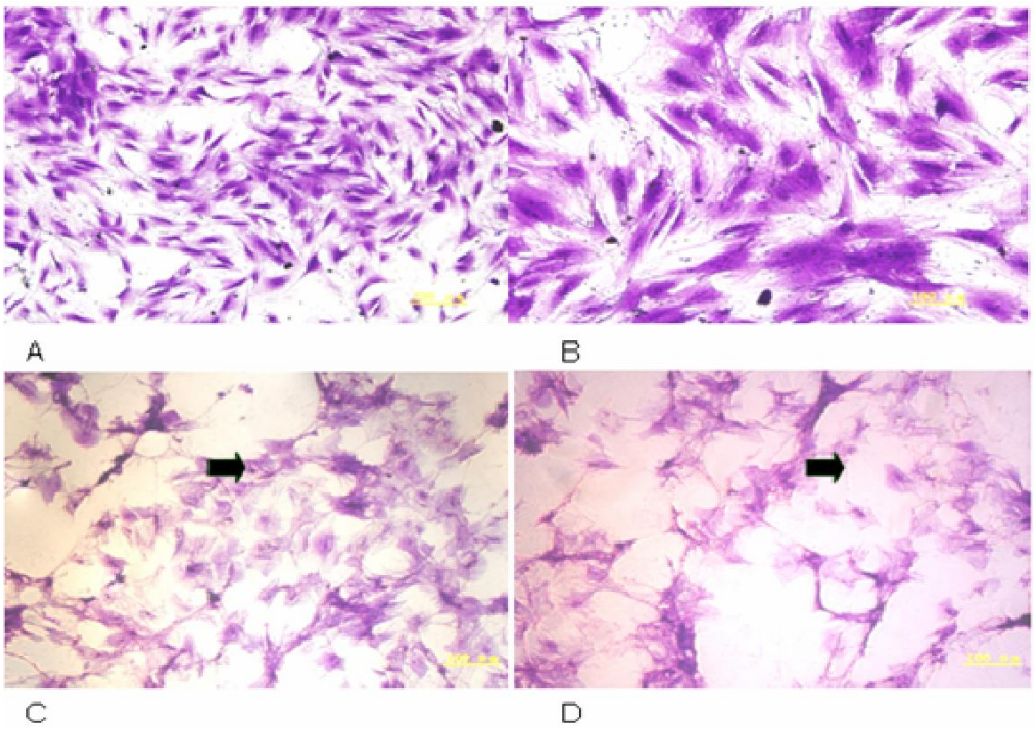

Figure 1. MSCs morphology after crystal iolet staining. (A) 2 Gy radiation (×100), (B) 4 Gy radiation (×200), (C) 8 Gy radiation (×200), (D) 12 Gy radiation (×200).

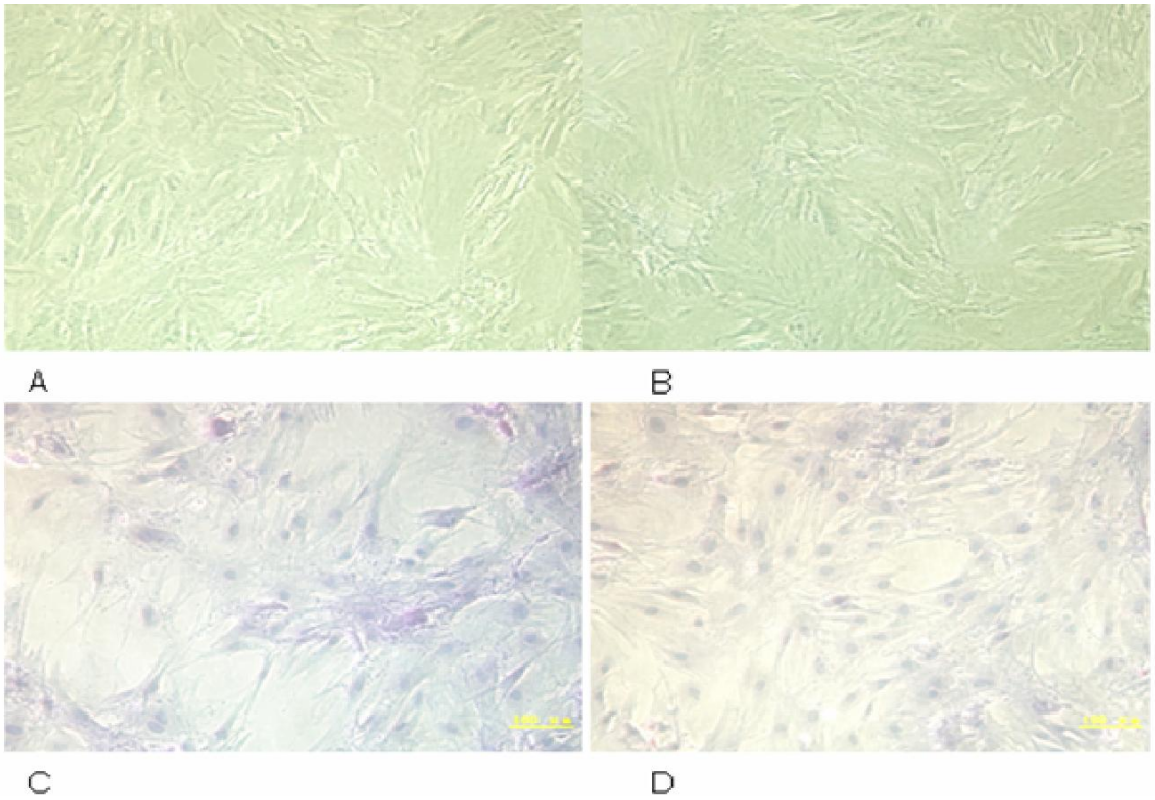

Figure 2. MSCs morphology after trypan blue staining. (A) 2 Gy radiation (×400), (B) 4 Gy radiation (×400), (C) 8 Gy radiation (×200), (D) 12 Gy radiation (×200).

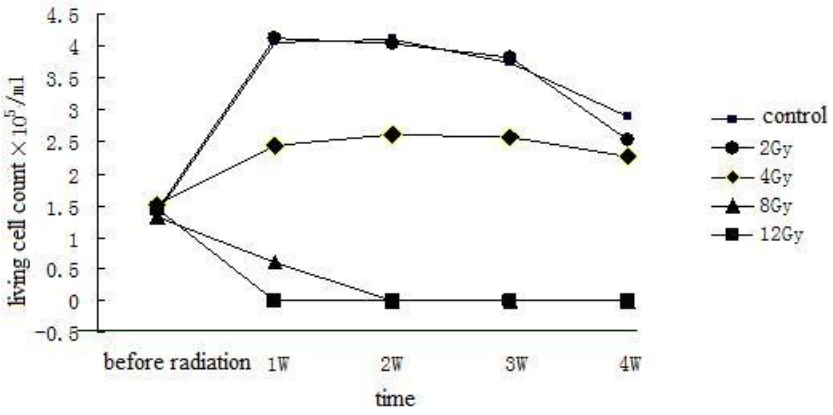

Figure 3. Trend in cell viability.

Table 3. Viable cell count (×10^5/ml, x ± s).

Group	Before radiation	Radiation (1W)	Radiation (2W)	Radiation (3W)	Radiation (4W)
Control	1.39 ± 0.27	4.07 ± 0.78	4.11 ± 0.74	3.74 ± 0.61	2.89 ± 0.35
2 Gy	1.42 ± 0.31	4.13 ± 0.81	4.05 ± 0.65	3.82 ± 0.63	2.54 ± 0.28
4 Gy	1.51 ± 0.36	2.45± 0.37#	2.61 ± 0.38#	2.58 ± 0.33#	2.27 ± 0.39
8 Gy	1.32 ± 0.22	0.6± 0.11	0	-	-
12 Gy	1.44 ± 0.35	0	-	-	-

#p < 0.05 s. control and 2 Gy radiation groups, P<0.05 s. control group.

Table 4. MSCs iability (%, x ± s).

Group	Before radiation	Radiation (1W)	Radiation (2W)	Radiation (3W)	Radiation (4W)
Control	93 ± 3	293 ± 47	296 ± 49	269 ± 35	208 ± 33
2 Gy	92 ± 3	291 ± 39	285 ± 44	271 ± 39	179 ± 27
4 Gy	94 ± 2	162 ± 28#	173 ± 31#	170 ± 29#	150 ± 16
8 Gy	93 ± 2	45 ± 7	0	-	-
12 Gy	91 ± 3	0	-	-	-

#p < 0.05 s. control and 2 Gy radiation groups, p < 0.05 s. control group.

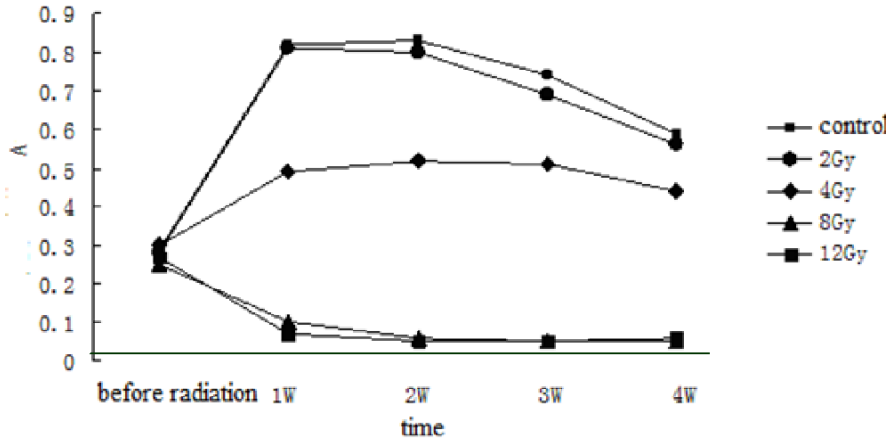

Figure 4. Trend in cell proliferation.

Table 5. Absorbance alue of different groups (x ± s).

Group	Before radiation	Radiation (1W)	Radiation (2W)	Radiation (3W)	Radiation (4W)
Control	0.29 ± 0.05	0.82 ± 0.13	0.83 ± 0.16	0.74 ± 0.12	0.59 ± 0.08
2Gy	0.28 ± 0.04	0.81 ± 0.15	0.80 ± 0.12	0.69 ± 0.11	0.56 ± 0.07
4Gy	0.30 ± 0.06	0.49 ± 0.09#	0.52 ± 0.08#	0.51 ± 0.07#	0.44 ± 0.06
8Gy	0.25 ± 0.03	0.10 ± 0.03	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
12Gy	0.27 ± 0.04	0.07 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.01

#p < 0.05 s. control and 2 Gy radiation groups.

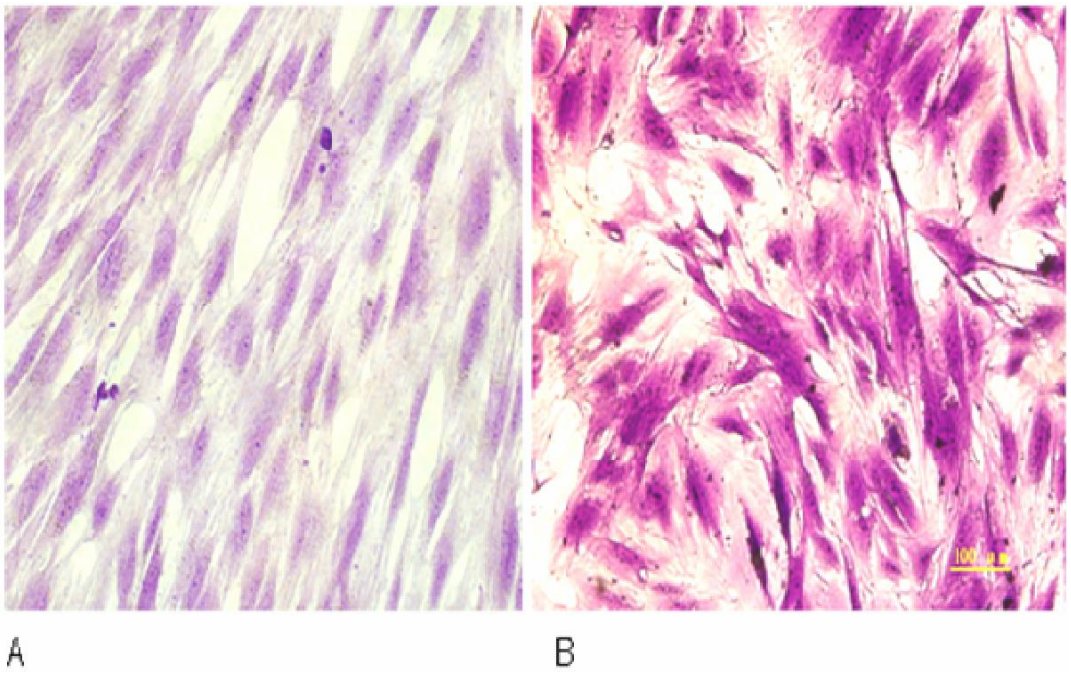

Figure 5. (A) Effect of 5-aza treatment on MSCs morphology in group A; (B) Effect of 5-aza treatment on MSCs morphology in group B.

The expression of myocardial-specific protein, C-TNT and -MHC was not detected in the cells of group B (Figure 6). The expression of -MHC (IOD, 0.36) and C-TNT (IOD, 0.21) in the cells of group A was detected after the 5-Aza treatment (Figure 6).

DISCUSSION

It is well documented that MSCs have the ability to differentiate osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, adipocytes and other mesenchymal phenotypes. Because of their multiple differentiation or plasticity property, this is especially important for MSCs that could possibly repopulate other tissues than the bone marrow (Wang and Li, 2007).

In this study, we found that DNA damage was induced in a linear dose-dependent way by 60Co -rays. The data regarding viable cell counts showed a significant decrease in the MSCs viability index compared to control. These changes were dose and time dependent. This observation was confirmed by the MTT and trypan blue assays. Our results demonstrate that high dose (12 Gy) of radiation could accelerate cell death. In contrast, low dose (4 Gy) of radiation did not significantly affect MSCs. However, radiation may eventually affect cell growth, proliferation, viability and migration capacity of MSCs. In addition, radiation not only inhibited cell-cycle progression but also acted as a promoter of apoptosis.

MSCs have a large capacity for self -renewal while maintaining their multipotency. Like their hematopoietic counterparts, the differentiation of MSCs involves multi-step cell lineages controlled by bioactive factors existed in the local micro-environment or supplied in the culture environment of *ex vivo* cultivated cells. Nevertheless,

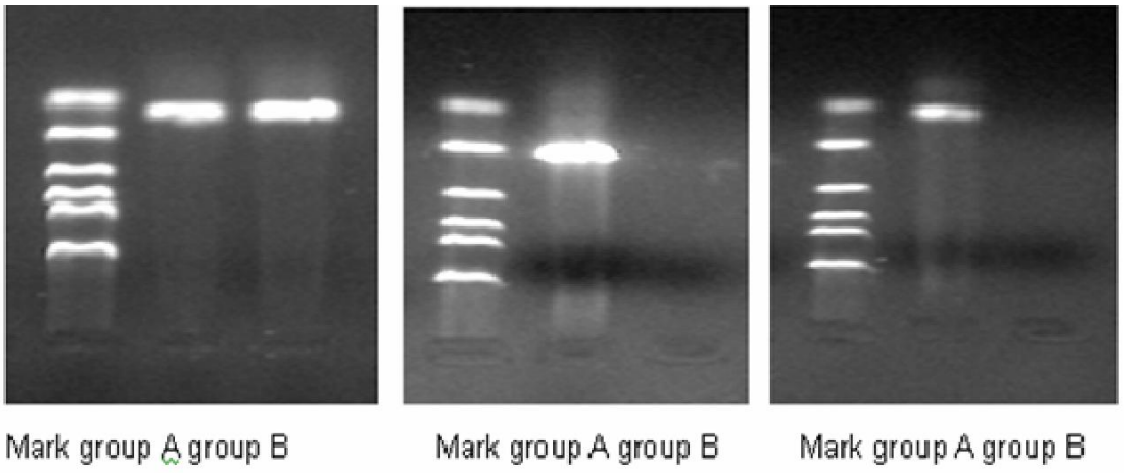

Figure 6. Effect of 5-aza treatment on expression of C-TNT and -MHC mRNA in MSCs cells.

unlike their hematopoietic counterparts, MSCs can be ease of isolation and expanded highly in culture. Furthermore, MSCs display genetic stability, reproducible attributes from isolate to isolate, reproducible characteristics in widely dispersed laboratories and compatibility with tissue engineering principles (Pittenger et al., 1999; Lodie et al., 2002; Gronthos et al., 2003).

5-azacytidine (5-aza), sold under the trade name Vidaza, is a chemical analogue of cytidine, a nucleoside present in DNA and RNA. Cells in the presence of azacitidine incorporate it into DNA during replication and RNA during transcription. The incorporation of azacytidine into DNA or RNA inhibits methyltransferase thereby causing demethylation in that sequence, affecting the way that cell regulation proteins are able to bind to the DNA/RNA substrate. Inhibition of DNA methylation occurs through the formation of stable complexes between the molecule and with DNA methyltransferases, thereby saturating cell methylation machinery.

Cardiac troponin T (cTnT), a thin-filament contractile protein present in high concentrations in the myocardium but usually not in other tissues, is released rapidly after myocardial injury in direct proportion to the extent of injury. It persists in the serum for several days, probably as a result of ongoing release from the heart, but is not present in the serum following nonmyocardial muscle or other tissue damage (Gerhardt et al., 1991).

Expression of alpha- and beta-myosin heavy chain (MHC), the two functionally distinct cardiac MHC isoforms is species-dependent and tightly controlled by develop-mental and hormonal factors (Everett et al., 1984; Allen and Leinwand, 2001). Relative expression levels of these isoforms can be altered in disease states such as cardiac failure or hypertrophy (Nadal- Ginard and Mahdavi, 1989). For example, in failing adult mouse hearts, a shift from the normally predominant alpha- MHC toward beta-MHC is often observed (Harada et al., 1999; Jayakumar et al., 2010). Similarly, up-regulation of beta-MHC transcription can serve as an early and sensitive marker of cardiac hypertrophy (Jones et al., 1996).

The expression of myocardial-specific protein, C-TNT and -MHC was not detected in the cells of group B (Figure 2B). The expression of -MHC (IOD, 0.36) and C-TNT (IOD, 0.21) in the cells of group A was detected after the 5-Aza treatment (Figure 4B). A possible explanation was that 60Co radiation affected or inhibited the ability of MSCs to differentiate to myocardium.

In this study, we demonstrate that middle dose (4 Gy) of irradiation induces morphological changes, as well as alteration in both proliferation and differentiation potentials of MSCs. 5-azacytidine treatment did not successfully induce the differentiation of MSCs to myocardium, A possible explanation was that high-dose (8 and 12 Gy) of 60Co radiation affected or inhibited the ability of MSCs to differentiate to myocardium.

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