In vitro and *in vivo* Biological Responses of Proton Irradiation from MC-50 Cyclotron

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Abstract - In this study, we investigated the biological damage and stress responses induced by ion beam (proton beam) irradiation as a basis for the development of protective measures against space radiation. We examined the biological effects of proton beam produced by MC-50 cyclotron at KIRAMS on the cultured cells and mice. The proton beam energy used in this study was 34.9 MeV and the absorption dose rate for cells and mice were 0.509 Gy sec^{-1} and 0.65 Gy sec^{-1}, respectively. The cell survival rates measured by plating efficiency showed the different sensitivity and dose-relationship between CHO cells and Balb/3T3 cells. HGPRT gene mutation frequency in Balb/3T3 was 15×10^{-6} Gy^{-1}, which was similar to the reported value of X-ray. When stress signaling proteins were examined in Balb/3T3 cells, IκB-α decreased markedly whereas p53, phospho-p53, and Rb increased after proton beam irradiation, which implied that the stress signaling pathways were activated by proton beam irradiation. In addition, cellular senescence was induced in IMR-90 cells. In the experiments with C57BL/6 mouse, the immune cells (white blood cells, lymphocytes) in the peripheral blood were greatly reduced following proton beam irradiation whereas red blood cells and platelets showed relatively little change. These results can be utilized as basic data for studying the biological effects of proton beam using MC-50 cyclotron with respect to proton therapy research as well as space radiation research.

Key words : Proton, Radiation, Cyclotron, Cell signaling, Immune

INTRODUCTION

The biological effect of space radiation is a new field of radiation biology, because human beings will explore more into space in this century. The charged particles are the main resources of space radiation outside the atmosphere. Proton comprises about 85% of space radiation and the rest 15% is composed of α-particle, heavy particle, and electron (Todd 2003). Therefore, it is very important to study the biological effects and countermeasures of charged particles for the evaluation of health problems and radiation protection during space exploration (Kennedy and Todd 2003). Although the biological effects of charged particles are being studied extensively in some countries including USA, Japan and China (Miller 2003), little or no studies have been reported in Korea. Therefore, we aimed to obtain the basic data on the biological effects of high energy proton beam and to investigate the possible application of MC-50 cyclotron (KIRAMS, Korea) proton beam as a model for space radiation research. In this study, we investigated the oxidative damage by proton beam irradiation in cell culture system and in mice.

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MATERIALS AND METHODS

1. Animals

The 7-weeks old female C57BL/6 mice were purchased

from Orient Inc. (Charles River Technology; Seoul, Korea), and were quarantined and adapted for 1 week before the experiment. Mice were housed in polycarbonate cages and were fed with standard animal diet and water *ad libitum*. Mice were kept at 22～24°C with a 12 h light-dark cycle. All animal experiments were approved by institutional animal care and use committee at Korea Atomic Energy Research Institute.

2. Cell culture

CHO (Chinese Hamster Ovary) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Balb/3T3 mouse fibroblast cells were cultured at the same condition as CHO cells except that 10% bovine calf serum was supplemented instead of 10% fetal bovine serum. IMR-90 human lung fibroblast cells were cultured in Minimal Essential Medium supplemented with 10% fetal bovine serum and antibiotics mentioned above. All cell lines were purchased from ATCC (Manassas, VA, USA).

3. Proton beam irradiation of cultured cells and mice

Proton beam (34.9 MeV) irradiation was performed using MC-50 cyclotron at Korea Institute of Radiological and Medical Sciences, Seoul, Republic of Korea. Cultured mammalian cells (CHO, Balb/3T3, IMR-90 cells) were irradiated in the polystyrene T-shape culture flask (5 cm × 5 cm) with 1～8 Gy of proton beam. For irradiation of C57BL/6 mice, they were confined in the acryl cage to minimize their movement and irradiated with 1～4 Gy of proton beam. The absorbed dose per current was calculated to be 0.509 Gy nA^{-1} for cultured cells and 0.65 Gy nA^{-1} for mice. For all irradiation practices, the beam current was 1 nA and total absorbed dose was adjusted by irradiation time.

4. Determination of survival rate and HGPRT mutation frequency

Proton beams were irradiated at doses of 1～6 Gy for CHO cells and at 0～4 Gy for Balb/3T3 cells. The culture medium was replaced with fresh medium within 30 minutes to minimize the effects of putative radioisotopes formed by radiation-activation.

To determine the survival rate, cells were detached from culture dish with Trypsin-EDTA at 30 hours after proton irradiation, plated to 6-well culture plate at 200 cells per well, and further cultured for 8 days. Then plates were washed with PBS and the colonies formed were counted after staining with 0.1% methylene blue (in 70% ethanol). The plating efficiency (PE) was calculated as the number of colonies divided by 200, and the survival rate was calculated as the PE of test group divided by PE of control group.

The mutation frequency of hypoxanthine guanine phosphoribosyl transferase (HGPRT) gene in CHO cells were determined as previously described (Cherubini *et al.* 2002). CHO cells irradiated with proton beams were cultured for 6 days, subculturing at third day. After trypsinization and counting, cells were plated to 100 mm culture dish (10^6 cells per dish) in media containing 5 μg ml^{-1} 6-thioguanine (6-TG). In addition, cells were plated to 6-well plates (200 cells per well) to determine the PE as described above. All culture plates were incubated for 8 days to allow colnony formation. The colonies formed were stained with methylene blue and counted as described above. The mutation frequency per 1 million cells of each group was calculated as the number of colonies of 6-TG treated plate divided by PE .

5. Detection of stress response proteins by Western blotting

Balb/3T3 mouse fibroblast cells were cultured in DMEM supplemented with 10% bovine calf serum. Cells were irradiated with 0, 1, 2, 4 Gy proton beam and the media was replaced with fresh media within 30 minutes after irradiation. Cells were harvested at 2 and 4 hours after irradiation, and were lysed in 100 μl of cell lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM AEBSF, 0.8 μM aprotinin, 20 μM leupeptin, 40 μM bestatin, 15 μM pepstatin, 14 μM E-64) for 30 min on ice. The cell lysates were centrifuged at 12,000 g for 20 min at 4°C and the supernatant solutions were collected. The protein concentration of the supernatant solution was determined by BCA method, and 20 μg protein was analyzed by western blotting. The primary antibodies were β-actin rabbit IgG, anti-phospho-Rb rabbit monoclonal IgG, anti-phospho-p53

rabbit IgG, anti-p53 mouse IgG (Cell Signaling Technologies Inc., Danvers, MA, USA) and anti-Rb rabbit monoclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and the secondary antibodies were HRP-linked anti-rabbit IgG or HRP-linked anti-mouse IgG (Cell signaling Technologies Inc., Danvers, MA, USA). The antibodies were used after dilution (1 : 200 to 1 : 1000).

6. Determination of senescence-associated β-galactosidase (SA-β-gal) in IMR-90 cells

IMR-90 human lung fibroblast cells cultured in 25 cm^2 T-flask at about 80% confluence were irradiated with proton beam. The cells were then detached by trypsinization and plated in 96-well plates (5×10^3 cells per well) and in 6-well plates (2×10^4 cells per well). Forty-eight hours later, senescence-associated β-galactosidase (SA-β-gal) activity was detected by a quantitative fluorometric method with di-β-D-galactopyranoside (FDG; Sigma-Aldrich Co., St. Louis, MO, USA) for 96-well plate cultures, and a qualitative staining method by 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-Gal) for 6-well plate cultures as previously described (Yang and Hu 2004). The FDG fluorescence was measured using a fluorometer (Mithras LB940; Berthold Technologies GmbH, Bad Wildbad, Germany) with an excitation at 485 nm and an emission at 535 nm. For X-gal staining, the stained cells were observed by an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) at ×100 magnification.

7. Measurement of hematological values of mouse peripheral blood

8-weeks old female C57BL/6 mice were divided into 4 groups (5 mice per group), and each group received the proton irradiation at 0, 1, 2, 4 Gy, respectively. For the measurement of hematological values, whole blood was collected to EDTA-tubes from the ophthalmic venous plexus of mice at 1 day before irradiation, and at 2, 8, 21 days after irradiation. The blood was well mixed for 30 minutes at room temperature and was analyzed by automatic hematology analyzer (HemaVET 850+, CDC Technologies Inc., Columbia, UK).

8. Statistical analysis

All data shown here represent the mean ± SD. Differences among the means were statistically analyzed using a two-tailed Student *t*-test with Microsoft Office Excel 2003 (Microsoft, Redmond, WA, USA). Differences with p values less than 5% were taken as significant.

RESULTS AND DISCUSSION

1. Effects of proton beam irradiation on the survival rate and mutation frequency in cells

To investigate the effects of proton beam irradiation on the cell growth or proliferative activity, the survival rates of CHO and Balb/3T3 cells exposed various doses of proton irradiation were determined by plating efficiency. The survival curve of CHO cells was sigmoid with steep decrease from 87.5% to 38.3% between 2 ~ 4 Gy and slow descent at doses lower than 2 Gy or higher than 4 Gy (Fig. 1A). On the other hand, Balb/3T3 cells showed exponential decrease in cell survival between 0 ~ 4 Gy (Fig. 1B). LD_{50} of Balb/3T3

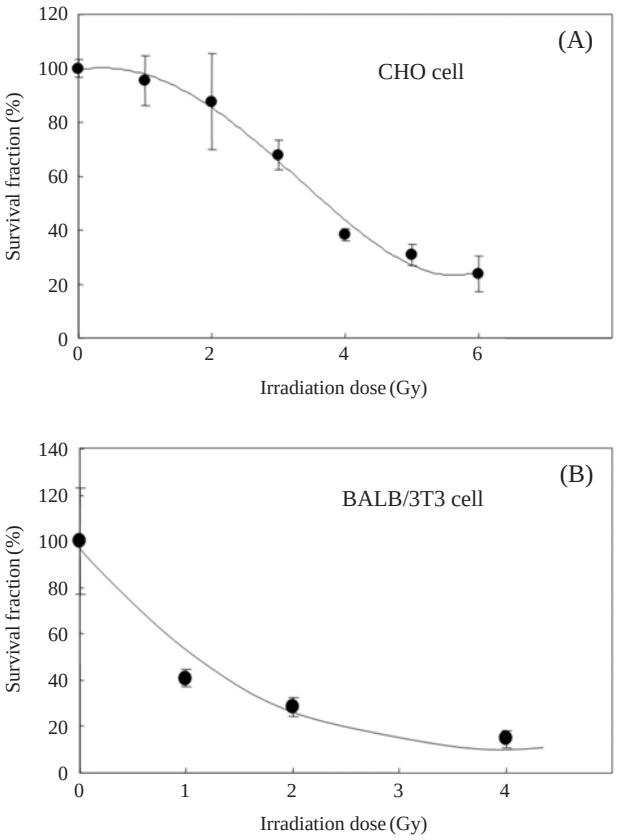

Fig. 1. Effects of proton beam irradiation on the plating efficiency. (A) CHO cells. (B) Balb/3T3 cells.

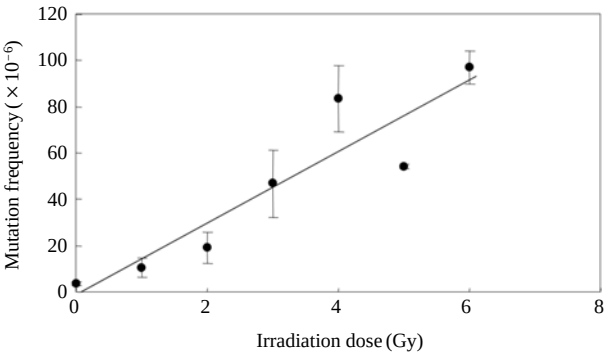

Fig. 2. Effects of proton beam irradiation on the HGPRT mutation frequency in CHO cells.

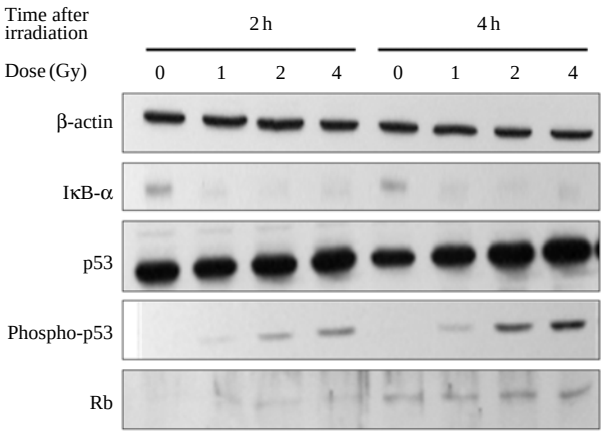

Fig. 3. Effects of proton beam irradiation on the expression and activation of cell signaling proteins in Balb/3T3 cells.

cells was lower than 1 Gy, whereas that of CHO cells was calculated to be 3.5 Gy indicating that Balb/3T3 cells are more sensitive to proton irradiation than CHO cells.

The mutation in hypoxanthine guanine phosphoribosyl transferase (HGPRT) gene is a widely-used biomarker in radiation biology since the mutants can be easily selected using the drug 6-thioguanine (Diamond *et al.* 1996). In our study, CHO cells were assayed for the induction of HGPRT gene mutation 6 days after proton beam irradiation. The mutation frequency was 3.6×10^{-6} in naïve CHO cells and increased linearly with the irradiation dose, reaching 97×10^{-6} at 6 Gy (Fig. 2). The increased mutation rate per dose was 15×10^{-6} per Gy.

The electromagnetic radiations such as X-rays and γ-rays are known to induce mutation by linear quadratic functions whereas the particle irradiations such as proton, neutron, heavy ion beams shows mutation induction in exponential increase (Hall and Giaccia 2012). Also the relative biological effectiveness (RBE) of proton beam irradiation is reported to be similar to those of X-rays or γ-rays (Yang 1999). In our results, the mutation frequency increased linearly to the dose of the proton beam irradiation and the mutation frequency was similar to that of X-rays (10×10^{-6} Gy^{-1}) and was lower than that of α-particles (50×10^{-6} Gy^{-1}), which were in good agreement with previous reports.

2. Effects on oxidative stress-related signaling in Balb/3T3 cells

The cellular damage induced by irradiation activates the signaling pathways leading to the repair of damage, cell cycle arrest, or cell death (Belli *et al.* 2002). To examine the effects of proton beam irradiation on stress signaling, the expression and phosphorylation of several signaling proteins in Balb/3T3 fibroblast cells were analyzed (Fig. 3). The levels of IκB-α were decreased 2 and 4 hours after irradiation, which indicated the degradation of IκB-α and the subsequent activation of NF-κB, which is reported to be important mediator of radiation-induced adverse effects such as inflammatory responses (Lee *et al.* 2011). The protein level of p53 was increased at 4 hours after proton beam irradiation. The phosphorylation of p53 was evident at 2 and 4 hours after irradiation and showed dose dependency. Rb was slightly induced at 2 hours and 4 hours after irradiation. p53 is well-known to be activated by DNA lesions and induces cell cycle arrest by cyclin-dependent kinase inhibition (Dulić *et al.* 1994). Activated Rb is also involved in the cell cycle arrest when DNA is damaged via p21 upregulation (Das *et al.* 2005). Therefore, our results suggest that MC-50 cyclotron proton beam irradiation may have induced significant DNA damages and subsequent responses including stress signaling and cell cycle arrest.

3. Induction of cellular senescence by proton irradiation in IMR-90 cells

Senescence-associated β-galactosidase (SA-β-gal) is a well-known biomarker of replicative or stress-induced premature senescence *in vitro* and *in vivo* (Itahaha *et al.* 2007). Also it is reported that ionizing radiation induces cellular senescence (Hong *et al.* 2010). Therefore, we investigated the effects of proton beam on the induction of cellular sene-

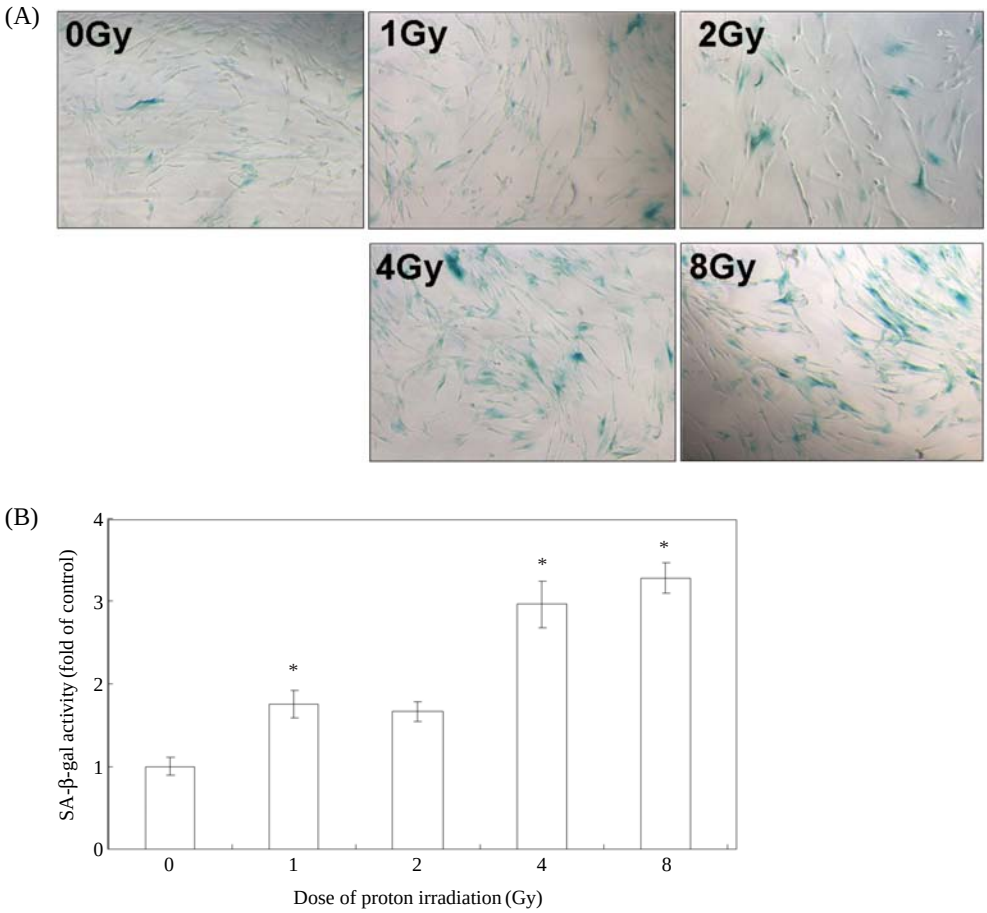

Fig. 4. Induction of senescence-associated β-galactosidase activity by proton beam irradiation in IMR-90 cells. (A) Microscopic observation after staining with X-gal. (B) Fluorometic activity assay with FDG. *Significantly different from non-irradiated group ($p < 0.001$).

scence in IMR-90 human lung fibroblast cells. As shown in Fig. 4A, IMR-90 cells irradiated with proton beam showed increased X-gal staining, which indicated the increase of SA-β-gal activity by proton beam irradiation. This result was further confirmed by fluorometric activity assay with FDG, which demonstrated the dose-dependent increase of SA-β-gal activity (Fig. 4B). These results suggest that proton beam irradiation induced cellular senescence in IMR-90 cells.

4. Effects of proton irradiation on immune system in C57BL/6 mice

It is reported that the immune and hematopoietic system is greatly affected by space radiation and 1.0 Gy of solar proton can be lethal to the crew members (Parsons and Townsend 2000; Epelman and Hamilton 2006). Therefore, we investigated the effects of MC-50 cyclotron proton beam on the immune system in C57BL/6 mice. Female 10 weeks old C57BL/6 mice were irradiated with 0～4 Gy of proton beam. The hematological values of the peripheral blood were measured 1 day before and 2, 8, and 21 days after irradiation.

Two days after irradiation, the white blood cells and lymphocytes were reduced dose-dependently to 30～58% and 28～58% of the pretest values, respectively (Fig. 5). These cells were partially recovered 8 days after irradiation, but the recovery was slower at higher doses of irradiation. In contrary to the WBC and lymphocytes, the red blood cells and platelet numbers were less affected by proton beam irradiation (Fig. 5). Temporary decreases of RBC in 4 Gy groups and platelet in 4 and 8 Gy groups were observed at 8 days after IR. These results showed that 1～4 Gy of MC-50 cyclotron proton beam induced immune suppression in a dose-dependent manner, and the recovery from this suppression is slower in higher doses. In contrast, RBC and platelet

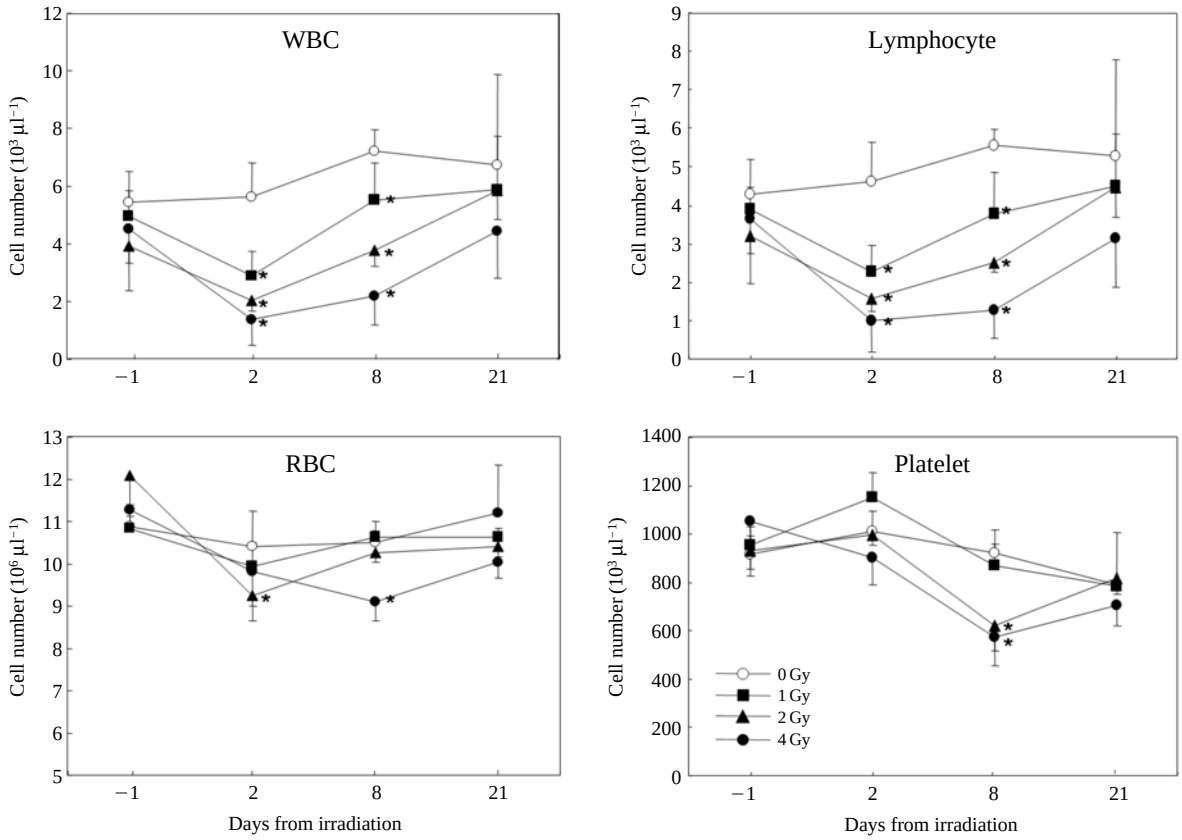

Fig. 5. The effects of proton beam irradiation on the hematological values of peripheral blood of C57BL/6 mice. The whole blood from control or irradiated mice were analyzed for the number of white blood cells (WBC), lymhocytes, red blood cells (RBC), and platelets. *Significantly different from non-irradiated group at each time point ($p < 0.05$).

numbers were affected more slightly within the period (21 days) observed in this study.

CONCLUSION

In this study, we observed that the irradiation of proton beam generated by MC-50 cyclotron elicited several biological effects *in vitro* and *in vivo*. The plating efficiency reduction and HPRT gene mutation were observed in CHO cells. Several intracellular signaling pathways including NF-κB, p53 and Rb were activated in Balb/3T3 mouse fibroblast cells. Cellular senescence was induced in IMR-90 human lung fibroblast cells. Also the depression of immune system was observed in C57BL/6 mice. These results showed that MC-50 cyclotron proton beam can elicit the clear biological responses comparable to those induced by X-ray or γ-rays, and provided the basic data for further investigation on biological effects of proton beam irradiation. Therefore, our results can provide basic biological data for using MC-50 cyclotron beam as a useful model for space radiation research as well as proton therapy research.

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