

Assessment of mouse BMMNC DNA damage with a two-tailed comet assay after X-ray and carbon ion total body irradiation

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Abstract The two-tailed comet assay (2T-comet assay) is a method for simultaneously evaluating DNA single-strand breaks (SSBs) and double-strand breaks (DSBs). In the present study, the endonuclease DNase I and hydrogen peroxide were used to induce DSBs and SSBs in bone marrow mononuclear cells (BMMNCs) from mice, and the damaged DNAs were assessed with a 2T-comet assay. The results demonstrated that this method can detect and discriminate between BMMNC DNA SSBs and DSBs simultaneously. Using this method, we studied DNA damage in BMMNCs from female BALB/c mice after total body irradiation with X-rays and carbon ions. The results indicated that these two types of radiation induced serious DNA damage in BMMNCs in a dose-dependent manner. The DNA damage induced by carbon ions was more severe than that induced by X-rays at the same dose, and a high dose of carbon ion radiation was more likely to cause death in mice. The DSBs and SSBs induced by X-rays were the highest on the 3rd day post-IR. For carbon ion radiation, DSBs were the most serious on the 3rd day, while SSBs were more obvious on the 3rd day and 13th day post-IR.

The ratio of DSBs/SSBs was clearly related to the different types of radiation.

Keywords Two-tailed comet assay, carbon ion irradiation · X-ray irradiation · Bone marrow mononuclear cells · DNA damage

1 Introduction

Individuals who are exposed to ionizing radiation (IR), as in the case of patients receiving radiotherapy for cancer or astronauts exploring space, are at increased risk of developing secondary cancers and other health-related problems. Previous studies have indicated that exposure to γ -rays or ^{56}Fe heavy ions increases the incidence of acute myeloid leukemia (AML) and hepatocellular carcinoma (HCC) in mice [1]. Bone marrow (BM), the predominant site of hematopoiesis and the differentiation of blood cells, is extremely sensitive to IR, including irradiation by high-charge and high-energy (HZE) particles. Irradiation with even as little as 0.1 Gy X-rays can cause a significant increase in micronucleated reticulocytes in BALB/c and C57BL/6 mice [2]. Total body γ -irradiation causes both acute and long-term damage in hematopoietic stem and progenitor cells [3, 4]. Total body HZE iron ion irradiation was shown to significantly increase chromosomal damage in mouse BM cells [5]. Exposure to HZE particles can have even more detrimental effects on BM hematopoietic precursors and mature blood cells [6, 7] because HZE particles have a greater propensity for ionization and because they deposit large amounts of energy along their tracks; thus, they have a greater potential for causing damage to tissues. High-linear energy transfer (LET) radiation from HZE ions

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is one of the major risks during spaceflight beyond low Earth orbit (LEO), and it is also encountered on the ground. For example, HZE carbon ion radiation is used in cancer radiotherapy [8]. Disruption of hematopoietic homeostasis can result in hematologic disorders and impact the function of vital organs, and abnormalities in hematopoietic cells in the BM can be propagated to the successive blood lineages and result in leukemia. Therefore, it is important to understand the effects of exposure to IR on BM. Unlike the effects of conventional radiation (γ - or X-rays) on the BM, both the short- and long-term effects of HZE ion radiation on this site of hematopoiesis are less well understood.

Exposure to IR results in multiple effects on BM through DNA damage induction. Thus, a precise understanding of DNA damage is critical for the prevention and treatment of radiation-induced damage. DNA damage can be assessed by the immunocytochemical detection of phosphorylated H2AX (γ -H2AX) and alkaline single-cell gel electrophoresis (ASCGE). γ -H2AX is considered to be a biomarker for the detection of double-strand breaks (DSBs) [9]. However, this method requires highly specific antibodies and a large quantity of cells, and the instruments used in the experiments have a significant influence on the detection results. Moreover, this technique is not suitable for assessing single-strand breaks (SSBs), which are also an important DNA damage form. ASCGE is known for its ability to detect DNA damage at the single cell level. It can detect DNA damages sensitively in a short time, using just a few sample cells. Under alkaline conditions, alkaline labile sites, cross-links, and some DSBs are measured as SSBs [10]. It is clear that distinguishing between SSBs and DSBs is necessary and important. The two-dimensional or two-tailed comet assay (2T-comet assay) is a modification of the original comet assay. It combines denaturing and non-denaturing conditions in the same cell nucleoid. Using this technique, Enciso [11] assessed DNA fragmentation in spermatozoa and found that the 2T-comet assay is a reliable method for the simultaneous characterization of SSBs and DSBs in the same human spermatozoa. If this method can be applied to the evaluation of BMMNC DNA damage after IR, it would have important implications for radiation protection and pathology.

In the present study, we evaluated the 2T-comet assay in BMMNCs by inducing DNA SSBs with hydrogen peroxide (H_2O_2 , an efficient SSB producer) and DSBs with a restriction enzyme [11]. Using this method, we comparatively studied the DNA damage in BMMNCs from female BALB/c mice after X-ray and carbon ion total body irradiation (TBI). Studying the biological consequences of these types of radiations is significant for understanding the consequences of space missions and cancer therapy regimens.

2 Materials and methods

2.1 Animals

Female BALB/c mice (8–10 weeks) were purchased from Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Gansu Province, China), and maintained in ventilated plastic cages at a maximum of 10 mice per cage under standard vivarium conditions. After a 1-week acclimatization period, the animals were randomly divided into groups with 32 animals in each control and radiation dose group. Euthanasia was performed with 100% ether at the 1st, 3rd, 8th, or 13th day post-exposure for analyses. There were 8 mice/group/time point. The present studies were approved by the Animal Care Committee of the Institute.

2.2 Irradiation

2.2.1 X-ray irradiation

An animal was placed in a plastic tube with breathing holes for TBI with X-rays. X-ray irradiation was performed with a Varian Clinac 21EX platinum device (VARIAN, America) operated at 6 MeVp, and the dose rate was 1 Gy/min. The doses used for this experiment were 1, 4, and 6 Gy. Non-irradiated mice were used as controls (0 Gy).

2.2.2 Carbon ion irradiation

An animal was placed in a plastic tube with breathing holes for TBI with carbon ions generated from the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China); the energy of the carbon ion beam when it entered the body was calculated to be 80 MeV/u, corresponding to a linear energy transfer (LET) 31.6 keV/ μ m in water, and the dose rate was adjusted to be about 1 Gy/min. The doses used for this experiment were 1, 4, and 6 Gy at the entrance region of the beam. Non-irradiated mice were used as controls (0 Gy).

2.3 Bone marrow mononuclear cells

The femora were harvested from the mice immediately after each group of mice was euthanized on the 1st, 3rd, 8th, or 13th day after irradiation or non-irradiation. Bone marrow cells were flushed from the bones into phosphate buffer saline (PBS) containing 0.1% heparin sodium using a needle and syringe. Then, the cells were centrifugated and resuspended in red blood lysing buffer, and after

10 min, they were centrifugated and washed with PBS twice and resuspended in PBS to obtain the BMMNCs.

2.4 DNA damage analysis

2.4.1 Two-tailed comet assay [12]

First, 10 μL containing about 5000 BMMNCs was mixed with 50 μL freshly preheated 1% low melting point agarose (LMP; Solarbio Life Sciences, China) in PBS at 37 °C. Then, 60 μL of the cell suspension was placed onto a glass slide, which was pretreated with a coating of a thin film of 1% standard agarose (Solarbio Life Sciences, China); it was then covered with a glass coverslip and stored at 4 °C for 5 min. As soon as the gel solidified, the coverslip was carefully removed. Then, the slides were submerged sequentially in two lysing solutions: lysing solution 1 [0.4 M Tris-HCl, 0.8 M dithiothreitol (DTT), 1% sodium dodecyl sulfate (SDS), pH 7.5] for 45 min at 4 °C, followed by lysing solution 2 [0.4 M Tris-HCl, 2 M NaCl, 0.05 M EDTA, 1% SDS, pH 7.5] for 45 min at 4 °C. Then, the slides were rinsed in TBE buffer (0.09 M Tris-borate, 2 mM EDTA, pH 7.5) for 5 min, transferred to an electrophoresis tank and immersed in fresh TBE buffer, and electrophoresed at 20 V (1 V/cm), 12 mA for 15 min. After neutral electrophoresis, slides were washed in 0.9% NaCl and nucleoids were unwound in an alkaline electrophoretic buffer (0.03 M NaOH, 1 M NaCl) for 5 min, then transferred to an electrophoresis chamber, and oriented 90° to the first run. The second electrophoresis was performed at 20 V (1 V/cm), 12 mA for 5 min in 0.03 M NaOH. Then, the slides were rinsed in a neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 min, washed in TBE buffer, and air-dried.

2.4.2 Induction of DNA SSBs

BMMNCs were harvested from the control mice as described above, and 1×10^5 cells concentrated in each sample. Then, the BMMNCs were incubated with 0.015, 0.03, and 0.15% H_2O_2 for 5 min at room temperature in order to induce the gradient SSBs. Next, the two-tailed comet assay was performed as described above.

2.4.3 Induction of DNA DSBs

The RNase-free endonuclease DNase I (Thermo Scientific, USA) was used to induce the DSBs. The BMMNCs from control mice were placed onto prepared glass slides, and after the 1-h lysis treatment for the 2T-comet assay described above, the slides were rinsed with reaction buffer [10 mM Tris-HCl (pH 7.5 at 25 °C), 2.5 mM MgCl_2 , 0.1 mM CaCl_2] for 20 min. Then, 20 μL of reaction buffer

containing 20 units of DNase I enzyme was placed on the slide, covered with coverslip, and incubated in a moist place at 37 °C for 3 or 6 min. After washing with TBE for 20 min, the slides were electrophoresed and the 2T-comet assay was continued as described above.

2.4.4 Comet assay analysis

Comet slides were stained with freshly prepared ethidium bromide stain (10 $\mu\text{g}/\text{mL}$; Sigma, USA), and images of about 100 cells for each sample were captured at a 200 \times magnification under a fluorescence microscope (Olympus-BX53, Japan) at an excitation wavelength of 525 nm, with three technical repeats. Two DNA damage parameters, tail moment (TM) and olive tail moment (OTM), were analyzed with CASPLab 1.2.2 software (Wroclaw, Poland) [13]. $\text{TM} = \text{Tail DNA}\% \times \text{Tail Length}$; $\text{OTM} = \text{Tail DNA}\% \times (\text{Tail Mean X} - \text{Head Mean X})$. “Tail DNA%” is the percentage of DNA in the tail, “Tail Mean X” is the center of gravity of DNA in the tail at the X coordinate, and “Head Mean X” is the center of gravity of DNA in the head at the X coordinate.

2.5 Statistical analysis

The data are presented as the mean \pm standard error. The significance of differences between irradiated groups and controls was assessed by Student's *t* test. A *p* value less than 0.01 was selected as a criterion for a statistically significant difference.

3 Results

3.1 Evaluation of the 2T-comet assay

The 2T-comet assay detected DNA SSBs and DSBs simultaneously. The first electrophoresis under neutral conditions was set to the X-axis, and the second electrophoresis run at a right angle to first electrophoresis direction under alkaline conditions was set to the Y-axis; the electrophoresis results are shown in Fig. 1. The TM and OTM of DSBs and SSBs were analyzed separately (Fig. 2).

In the BMMNCs treated with H_2O_2 , the yield of SSBs increased significantly as the H_2O_2 concentration increased, and there were no obvious DSBs (Fig. 2a). In the BMMNCs treated with DNase I, the TM and OTM of DSBs increased significantly as the time of enzyme treatment increased, while SSBs remained at the control level (Fig. 2b). In the cells treated with H_2O_2 and DNase I successively, the degree of DSBs (Fig. 2c) was not significantly different from the degree of DSBs in the cells treated with DNase alone (Fig. 2b), and the degree of SSBs

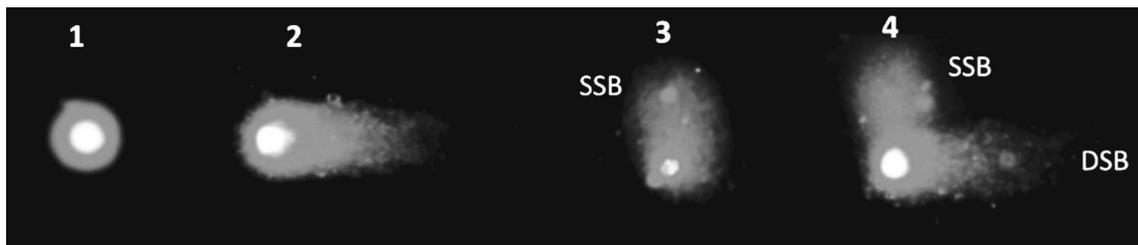


Fig. 1 Two-tailed comet assay detected different comet types as follows: 1, undamaged; 2, double-strand DNA breaks (DSBs); 3, single-strand breaks (SSBs); and 4, SSBs and DSBs

(Fig. 2c) was almost the same as the degree of SSBs in the cells treated with H_2O_2 alone (Fig. 2a).

These results showed that the 2T-comet assay could simultaneously detect BMMNC DNA SSBs and DSBs.

3.2 Effects of TBI on DNA damage in BMMNCs

The IR-induced DSBs and SSBs were simultaneously detected with the 2T-comet assay and analyzed separately with software. The results are shown in Fig. 3.

DNA damage induced by X-rays was time dependent and positively correlated with the dose. The most serious DNA DSBs and SSBs occurred on the 3rd day after X-ray irradiation (Fig. 3a, b, compared with the 1st, 8th, and 13th days, $p < 0.01$). Carbon ion irradiation also caused DNA damage in time- and dose-dependent manners. Similar to the results of X-ray irradiation, the yield of DSBs was highest on the 3rd day and then decreased (Fig. 3c, compared with the 1st, 8th, and 13th days, $p < 0.01$), but the results for SSBs after carbon ion irradiation were different. The TM and OTM increased significantly on the 3rd and 13th days compared with the results on the 1st and 8th days post-IR ($p < 0.01$, Fig. 3d). There were no results for the 13th day after 6 Gy carbon ion irradiation because 6 Gy carbon ion irradiation killed the mice starting on the 4th day after irradiation; 3, 6, and 1 mice died on the 4th, 7th, and 8th days, respectively, after 6 Gy carbon ion irradiation.

Compared with X-rays, carbon ion radiation caused more severe DNA damage, and the yield of DSBs was much higher than that of SSBs on the 1st, 3rd, and 8th days post-IR (Fig. 3c, d). The ratios of DSBs/SSBs induced by X-ray irradiation were lower than the ratios of DSBs/SSBs induced by carbon ions at the same time point (1st, 3rd, or 8th day), but on the 13th day, the ratios of DSBs/SSBs induced by X-ray irradiation were higher than the ratios of DSBs/SSBs induced by carbon ions. These results indicated that the DSBs/SSBs ratio was clearly related to the different types of radiation (Table 1).

4 Discussion

The 2T-comet assay is a rapid and sensitive method for simultaneously assessing DNA SSBs and DSBs in spermatozoa [11], and it has been used for genetic toxicology in spermatozoa [14, 15]. Using the 2T-comet technique, we detected SSBs and DSBs simultaneously in the same BMMNCs after H_2O_2 and DNase I treatment, and the results indicated that the 2T-comet technique is also capable of detecting SSBs and DSBs simultaneously in the same BMMNCs (Fig. 1).

There is an increasing need to understand the health effects of high-LET radiation due to the high potential for exposure to high-LET radiation during space missions and the growing utilization of heavy ion radiotherapy in medicine. The process of hematopoiesis in the BM controls the development of all blood lineages and is responsible for maintaining hematologic homeostasis. The hematopoietic system is extremely sensitive to IR exposure, and its damage can reduce radiation tolerance and increase the risk of developing secondary cancers and other health-related problems. To estimate the biological risks to the hematopoietic system of high-LET and low-LET IR, the DNA damage in BMMNCs from mice after X-ray and carbon ion TBI was studied using the 2T-comet method.

As expected, both the carbon ion and X-ray radiation-induced BMMNC DNA damages were positively correlated with the doses at the same time point post-IR (Fig. 3). Moreover, the DNA damage, especially the DSBs, induced by carbon ions (Fig. 3c) was more severe than that induced by X-rays (Fig. 3a). We also noted that a high dose of carbon ion IR was more likely to induce death in mice and may produce short-term effects. However, the results of the time effect indicated that the BMMNC DNA damages did not decrease continuously within the period of 13 days following irradiation with carbon ions or X-rays. The most serious DNA DSBs and SSBs occurred on day 3 post-X-ray IR (Fig. 3a, b). For carbon ion radiation, DNA DSBs were the most serious on the 3rd day, while DNA SSBs were more serious on the 3rd day and 13th day post-IR (Fig. 3c, d).

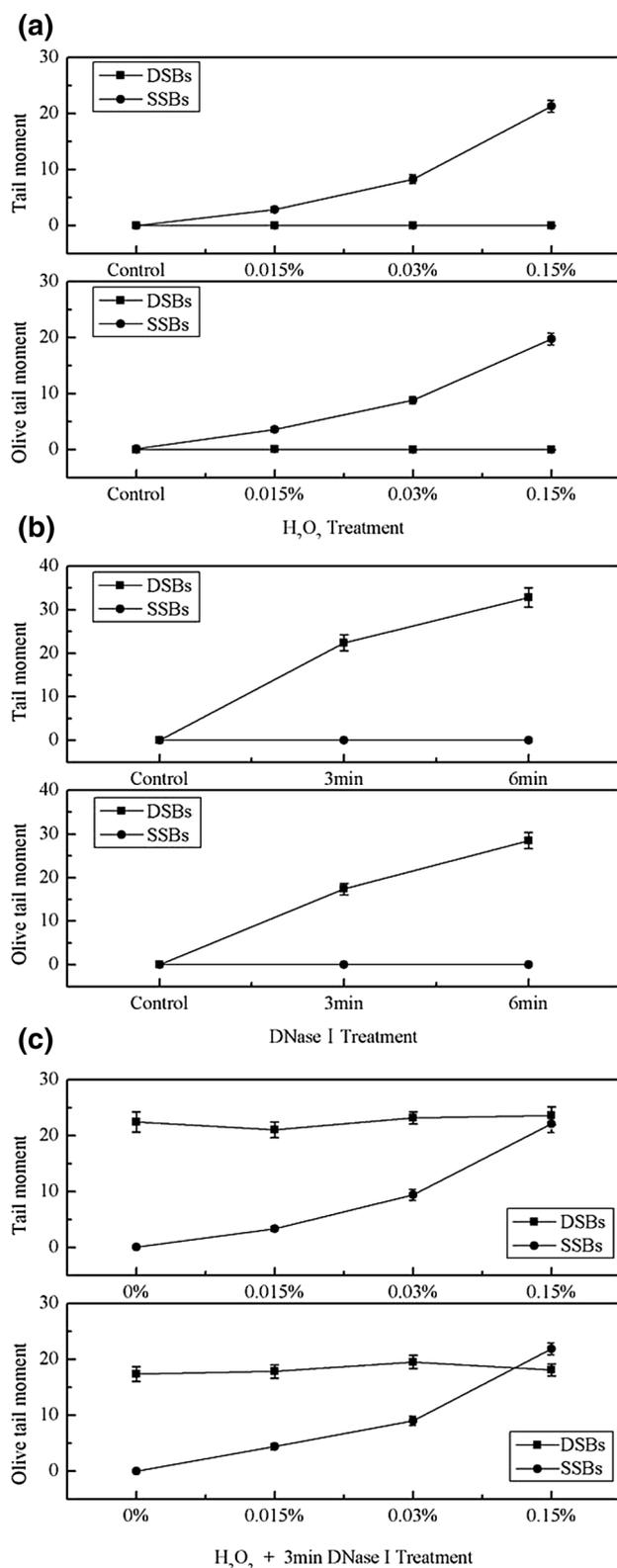


Fig. 2 Assessing DNA damage in mouse BMMNCs after different treatments with a two-tailed comet assay. DSBs = DNA double-strand breaks; SSBs = DNA single-strand breaks. **a** DNA damage in BMMNCs treated with different concentrations of H₂O₂. **b** DNA damage in BMMNCs digested with DNase I for different times. **c** DNA damage in BMMNCs treated with different concentrations of H₂O₂ and a 3-min DNase I digestion

It is well known that IR induces DNA damage by direct interactions between radiation and target macromolecules or by-products of water radiolysis. Due to its special ion track structure, high-LET IR causes more direct and indirect effects in cells than low-LET IR at the same dose. IR-induced DNA damage results in the activation of the DNA damage response (DDR), which represents a network of signaling pathways that regulate the response within cells to DNA damage. Numerous proteins have been found to participate in the DDR pathway. Cells within the BM often exhibit low levels of expression of many DDR proteins, such as ATM, ATR, and DNA-PKcs, suggesting that homologous recombination (HR)- and non-homologous end joining (NHEJ)-mediated repair are impaired and DNA damage repair activity is at low efficiency [16]. Furthermore, IR-induced reactive oxygen (ROS) and nitrogen (RNS) species could arise continuously for days and months after the initial exposure [17]. A 6.5 Gy γ -ray TBI induced a 4-week elevation in ROS production in mouse BMMNCs, but the peak occurred on the 3rd day post-IR [18], which was a similar trend as observed for DNA DSBs in BMMNCs (Fig. 3a, c). Chronic exposure to oxidative stress can lead to the accumulation of DNA lesions [19]. In addition, radiation-induced DNA damage frequently occurs in clusters, and the degree of clustering is determined by the radiation type; densely ionizing radiation such as carbon ions produces more clustered damage than sparsely ionizing radiation such as X-rays [20], and a certain type of clustered base damage can be transformed to SSBs and DSBs after base excision repair (BER) [21]. Together, the low DNA damage repair activity, DNA lesions induced by chronic oxidative stress, and DNA strand breaks produced by BER resulted in more serious DNA damage in BMMNCs on day 3 than on day 1 post-IR (Fig. 3a–d). Afterward, some of the damaged DNA was repaired and newly generated DNA damage (especially DSB) decreased because the levels of reactive species decreased gradually [18, 22]; in addition, some of the cells with serious DNA damage were cleared, resulting in decreased levels of DNA damage (Fig. 3). The quantity and type of DNA damage after IR are related to the yield of ROS: A small amount of ROS can induce SSBs, but a large amount of ROS is needed to induce DSBs [23, 24]. SSBs induced by carbon ion irradiation increased on the 13th day after IR

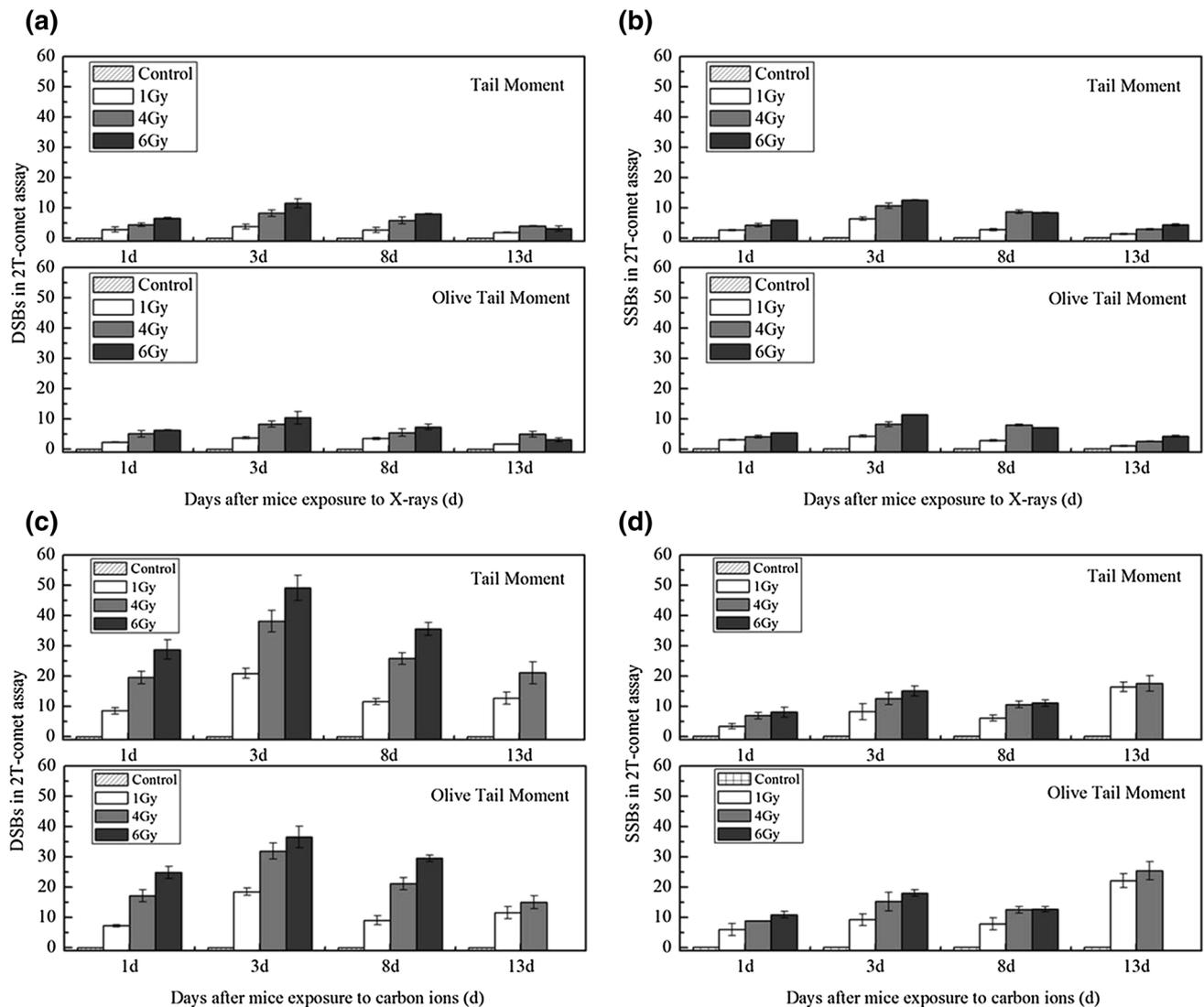


Fig. 3 DNA damage in mouse BMMNCs after total body X-ray and carbon ion irradiation. DSBs = DNA double-strand breaks; SSBs = DNA single-strand breaks. The data are presented as the mean \pm SE

($N = 6$ for 6 Gy carbon ion group on the 8th day; $N = 8$ for all the other groups). There were no results for the 13th day after 6 Gy carbon ion irradiation because the mice died

presumably because residual ROS mainly induced the SSBs (Fig. 3d) [17, 18].

BMMNC DNA SSBs and DSBs induced by carbon ion and X-ray IR could be easily and rapidly estimated simultaneously with the 2T-comet assay, and the ratios of DSBs/SSBs were also obtained (Table 1). The results indicated that the DSBs/SSBs ratio was obviously related to the radiation quality and could be used as a characteristic parameter of radiation quality [25]. It is possible that the DSBs/SSBs ratio, together with the DSB yield, could be used to determine the radiation quality, which is meaningful for mixed radiation fields, such as the radiation encountered during space flights.

5 Conclusion

A precise understanding of DNA damage is critical for the prevention and treatment of radiation-induced damage because exposure to IR results in multiple effects due to DNA damage. The 2T-comet assay could easily and rapidly estimate BMMNC DNA SSBs and DSBs simultaneously. This study on the 2T-comet assay indicated that both X-ray and carbon ion irradiation caused serious DNA damage in BMMNCs in time- and dose-dependent manners. Even on day 13 post-irradiation, DNA damage was still obvious. High-LET radiation (carbon ions) induced more severe DNA damage than that induced by low-LET radiation (X-rays) at the same dose. The ratio of DSBs/SSBs obtained by the 2T-comet assay was clearly related to

Table 1 Ratio of DSBs/SSBs in mouse BMMNCs after X-ray and carbon ion TBI

	Ratio of DSBs/SSBs					
	X-rays			Carbon ions		
	1 Gy	4 Gy	6 Gy	1 Gy	4 Gy	6 Gy
<i>TM</i>						
1d	1.09 ± 0.2	1.03 ± 0.09	1.10 ± 0.03	1.43 ± 0.18	2.22 ± 0.08	2.65 ± 0.13
3d	0.59 ± 0.07	0.77 ± 0.06	0.91 ± 0.08	2.27 ± 0.03	2.50 ± 0.39	2.73 ± 0.18
8d	0.98 ± 0.19	0.67 ± 0.08	0.94 ± 0.01	1.47 ± 0.16	2.06 ± 0.16	2.81 ± 0.08
13d	1.41 ± 0.02	1.38 ± 0.04	0.71 ± 0.12	0.57 ± 0.05	0.83 ± 0.15	– ^a
<i>OTM</i>						
1d	0.74 ± 0.06	1.23 ± 0.23	1.16 ± 0.03	2.11 ± 0.13	2.46 ± 0.05	3.09 ± 0.17
3d	0.86 ± 0.07	1.01 ± 0.13	0.91 ± 0.07	2.26 ± 0.12	2.54 ± 0.08	2.42 ± 0.13
8d	1.23 ± 0.14	0.68 ± 0.13	1.04 ± 0.01	1.47 ± 0.08	1.99 ± 0.13	2.66 ± 0.06
13d	1.52 ± 0.04	1.94 ± 0.03	0.72 ± 0.02	0.70 ± 0.1	0.86 ± 0.03	– ^a

The data are presented as the mean ± SE ($N = 6$ for the 6 Gy carbon ion group on the 8th day; $N = 8$ for all the other groups)

^aThere were no results for the 13th day after 6 Gy carbon ion irradiation because the mice died

the radiation quality and represents a potential characteristic parameter for radiation quality.

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