N-acetylcysteine protect lymphocytes and cytokines against heavy ion irradiation *via* counteracting the glutamate

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Abstract We evaluated the protective effect of *N*-acetylcysteine (NAC) on immunity system irradiated by $^{12}C^{6+}$ ion beam. Kun-Ming mice were whole-body irradiated by $^{12}C^{6+}$ ion at doses of 0, 0.5, 1, 1.5, 2, 2.5 and 3 Gy. The results showed that in saline group, the lymphocytes DNA double-strand breaks (DSBs), maleic dialdehyde, thymocytes number in G_0/G_1 and apoptosis percentage increased with dose increment, and the levels of interferon-γ, glutathione, superoxide radical (SOD) and natural killer cells activity decreased with dose increment. However, there were no significant changes in NAC-treated group. The data indicated that pre-treatment with NAC could significantly remove the ROS by counteracting the glutamate, decrease excessive lipid peroxidation reaction and SOD damages, and protect DNA, lymphocytes and cytokines against irradiation.

Key words *N*-acetylcysteine, $^{12}C^{6+}$ heavy ion, DNA damages, Oxidative stress, Lymphocytes and cytokines

1 Introduction

Humans are constantly exposed to ionizing radiations both from natural source like cosmic rays (during space travel), radioisotopes found in the earth crust and also from a wide variety of artificial sources. Currently, ionizing radiation finds extensive application such as power generation, developing new varieties of high-yielding crops, sterilization and enhancing the storage period of food materials (food irradiation), etc. With such a wide application of ionizing radiation, human exposure to these radiations has become inevitable. The main health concerns during exposure to radiations are poorly understood, although the risk of carcinogenesis and degenerative disease has been reported[1]. Several studies have reported that the cellular damage caused by ionizing radiation is predominantly mediated through free radicals and resultant reactive oxygen species (ROS)[2]. These primary radicals generate during the radiolysis of water react with molecules like oxygen producing secondary radicals (H_2O_2 and O^{2-}), which could damage vital cellular targets like DNA, proteins and membrane and ultimately lead to cell death and cancer[3]. Recently many researches have focused on the potential use of thiols as free radical scavengers to prevent oxidative damage.

Glutathione (GSH) is the principal intracellular thiol responsible for scavenging ROS and maintaining the oxidative balance in tissues. Cysteine and glutathione delivery compounds have been used to protect normal cells from redox imbalance[4]. One of the most widely thiol antioxidants uses is *N*-acetylcysteine (NAC), the NAC indirectly replenish GSH through deacetylation to cysteine, preventing oxidative damage through scavenging of ROS[5].

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One of the most radiosensitive systems of mammalian organism is immunity. The major role of thymus in more evolved vertebrates is to provide the appropriate microenvironment where T cells precursors (thymocytes) can mature. Natural killer (NK) cells function in innate immunity, is crucial for its capacity to combat viral infection and destroy cancer cells[6,7]. Low NK cell activity is associated with increased cancer risk. Interferon-γ (IFN-γ) is a pleiotropic cytokine that plays important roles in the regulation of inflammatory and immune responses[8-10].

Based on these observations about the radiation and ability of thiols, the protection of NAC in mice immunity system against heavy ion irradiation and the possible underlying mechanisms were investigated.

2 Materials and methods

2.1 Animals

Outbreed Kun-Ming mice (6−7 weeks) of half gender, weighing 23 g±1 g, were provided by Institute of Veterinarian in Lanzhou, Chinese Academy of Agricultural Sciences. They were randomly divided into 3 groups, the control, NAC-treated and saline-treated groups of 36 animals (6 in a subgroup). One hour before irradiation, NAC (200 mg/kg) was dissolved in saline (0.85%) and the saline of the same volume was given to the mice by celiac injections, respectively. This study was approved by the Animal Care Committee in the institute.

2.2 Irradiation

The carbon ion beam was provided by Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). The animal was whole-body plateau irradiated individually (energy of 284.7 MeV/u, linear energy transfer (LET) of 25.6 keV/μm and dose rate of 1 Gy/min), at the doses of 0, 0.5, 1, 1.5, 2, 2.5 and 3 Gy. This experiment was completed three times.

2.3 Levels of superoxide radical (SOD) activity, maleic dialdehyde (MDA), GSH and IFN-γ in serum.

After 2 hours irradiation, blood samples were collected in tubes from the orbital sinus, and then mice were sacrificed by cervical dislocation.

Thiobarbaturic acid (TBA) method of Hodges[11] was used to determine the lipid peroxidation by determining the amount of TBA reactive substances present. Commercial detection kits for MDA were carried out in an ultraviolet spectrophotometer (Nanjing Jiancheng, China).

SOD activity was determined by the spectrophotometric method[12] with the method of ρ-nitroblue tetrazolium chloride (NBT) to formazan.

GSH levels were measured by spectrophotometrically in the supernatant, at 412 nm, using 5,5′-dithiobis (2-nitrobenzoic acid) (ε_{412}=13,600$M^{-1}cm^{-1}$)[13].

The IFN-γ levels were detected by commercially available enzyme linked immunosorbent assays (ELISA) specific for mice (eBioscience San Diego, California, USA).

2.4 Comet assay of splenic lymphocytes

We used modified neutral comet assay procedure to provide quantitative measures of splenic lymphocytes DSBs[14]. Lymphocytes separation medium (LSM) (Shanghai Sangon Biological Engineering Technology and Service Co. Ltd, Shanghai, China) was used for isolation of lymphocytes using low-speed centrifugation (300×g) with time of 20 min. The professional software Comet Assay Software Project (Casp, Wrocław Poland) was used to analyse digitised images.

2.5 Levels of NK cells activity in spleen

NK cells activity was tested by means of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay[15].

2.6 Cycle and apoptosis of thymucytes

The thymocytes cycle and apoptosis were measured by Flow CytoMeter (Becton Dickinson). Cells were stained by propidium iodide (PI, Sigma-Aldrich, Missori, U.S.A.) only[16]. The data were acquired using FAC Scan software (Becton Dickinson, New Jersey, U.S.A.) and analyzed using the ModFit LT 3.0 software (Becton Dickinson).

2.7 Statistical analysis

Each value was expressed as the mean ±SD. An ANOVA analysis of variance was used to determine

the level of any statistically significant differences among NAC, saline and control groups. A *p*-level of 0.05 or less was selected as a criterion for a statistically significant difference.

3 Results and discussion

Heavy ion irradiation is well known to produce ROS[17]. In this study, the data of olive tail monment (OTM) of splenic lymphocytes increased with dose increment, and by 220% at dose of 3Gy compare to control in saline group in Figs.1a and 1b. Excessive DNA double-strand breaks (DSBs) meant the decrease of cells activity and increase of apoptosis. Our data showed that heavy ion irradiation had a dose-dependent increase of DSBs in saline group. Studies reported that DSBs were caused by ROS primarily induced by irradiation[18]. The high levels of DNA damages indicated that heavy ion irradiation could induce overactive ROS accumulation, and the levels of ROS increased with dose increment.

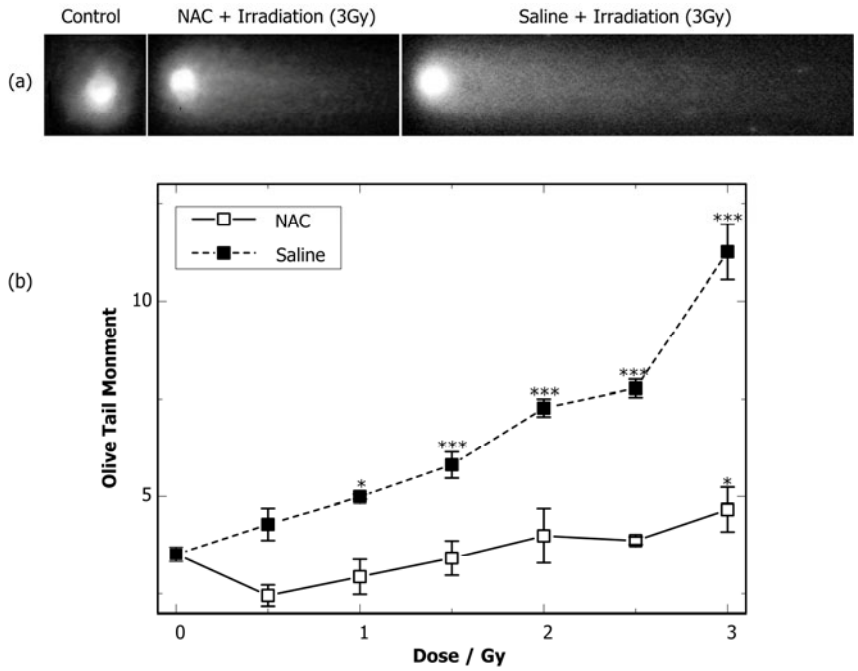

Fig.1 Effect of NAC on DNA damages of lymphocytes in spleen. (a) Photos of DNA fragmentation. (b) DNA double-strand breaks were showed by OTM (**p*<0.05, ***p*<0.01, ****p*<0.001 *vs.* controls).

Increased generation of ROS may trigger lipid peroxidation. MDA is an end product of the lipid peroxidation process which can be defined as oxidative deterioration of polyunsaturated lipids. In Fig.2a, there were high levels of MDA treated by saline and no significant increase in NAC group. The data showed high MDA concentrations in irradiation animals. Intracellular ROS levels are regulated by a series of antioxidative enzymes such as SOD. In Fig.2b, the levels of SOD significantly decreased with dose increment in saline group, and had no significant changes after NAC-treated. SOD activity received negative effect by ROS at high dose irradiation, and low levels of SOD had little effect on ROS regulation. The data indicated that the regulation of ROS *in vivo* was thrown into confusion, and the unmanageable ROS made the cells serious damages. And NAC could protect them against irradiation.

GSH antioxidant system plays crucial roles in counteracting ROS injuries. There are lots of reports that increasing cellular GSH level can protect cells against oxidative damage, while depleting GSH can augment such injury[19,20]. As there are dose-dependent discrepancies of DSBs induced by irradiation, the investigation of dose-dependent difference in GSH antioxidant system is of great importance. In this case, GSH levels significantly decreased with dose exposure to irradiation, and lowered decrease rate in NAC group in Fig.2c. Depletion of GSH may result in mitochondrial dysfunction[21] because electron transport complexes are susceptible to the attack of ROS *in vitro*[22]. It has been reported that GSH has an

important role in protecting against the mitochondrial damage induced by sustained exposure to ROS[4,23]. Furthermore, consumption of GSH was shown to be involved in the impairment of energy metabolism[24]. This may be due to decreased GSH levels, followed by ROS accumulation, and mitochondrial dysfunction. In our case, mitochondrial dysfunction and generation of ROS directly lead to oxidative stress. NAC used seems to be a GSH precursor rather than a ROS scavenger. Our data indicated that GSH could keep oxidative balance by removing the ROS and protect mitochondria through counteracting itself, and depleting GSH could augment cellular DSBs levels[25].

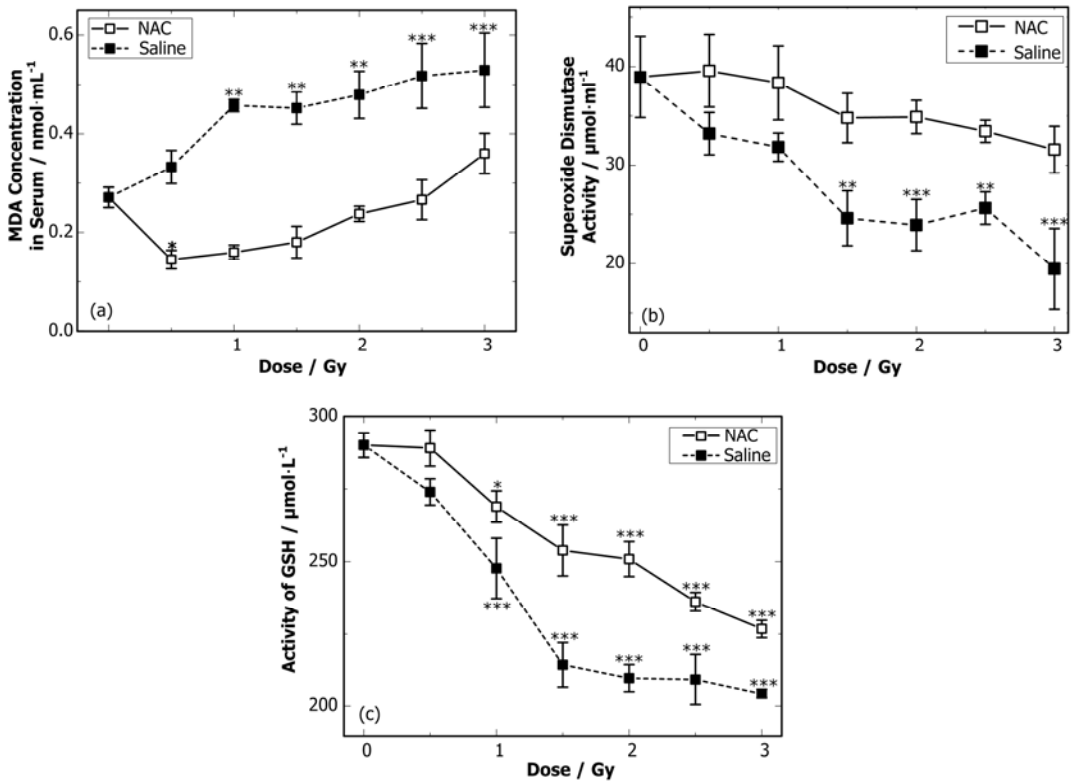

Fig.2 Capacity of NAC to scavenge ROS. Levels of ROS were showed by (A) MDA and (B) SOD activity. (C) NAC could remove ROS by counteracting the glutamate (*p<0.05, **p<0.01, ***p<0.001 *vs.* controls).

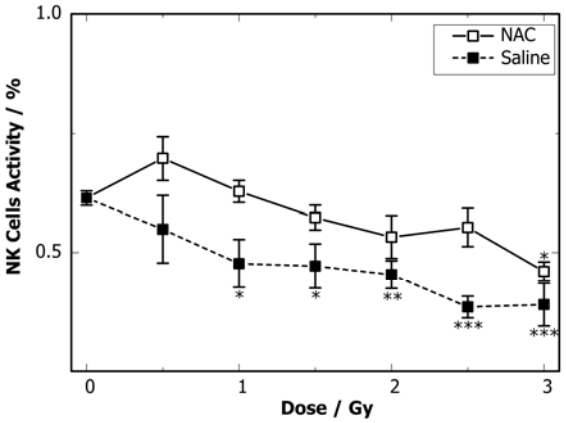

Fig.3 NAC protective effect on splenic NK cells activity expose to irradiation at doses of 0, 0.5, 1, 1.5, 2, 2.5 and 3 Gy (*p<0.05,**p<0.01,***p<0.001 *vs.* controls).

Immunological responses are mediated by immunocompetent cells and highly regulated by a complex network of cytokines. In our study, decline of NK cells and thymocytes activity induced by serious DSBs (Figs.3 and 4), may lead to decreases of responsiveness to IFN-γ (Fig.5). Activated NK and T cells could secrete IFN-γ. In turn, IFN-γ could induce ability of NK cells, T cells maturity, and inflammatory through ROS and NO production. In this study, low IFN-γ and high ROS levels could cause confusion of IFN-γ signaling and decrease of NK and T cells ability, ultimately lead to decline of immunity *in vivo*.

In general, whole-body $^{12}C^{6+}$ ion irradiation could induce serious DSBs by excessive and overactive ROS. Pre-treatment with NAC could significantly remove ROS by counteracting the glutamate, decrease excessive lipid peroxidation reaction and SOD damages, and protect organismic immunity against irradiation.

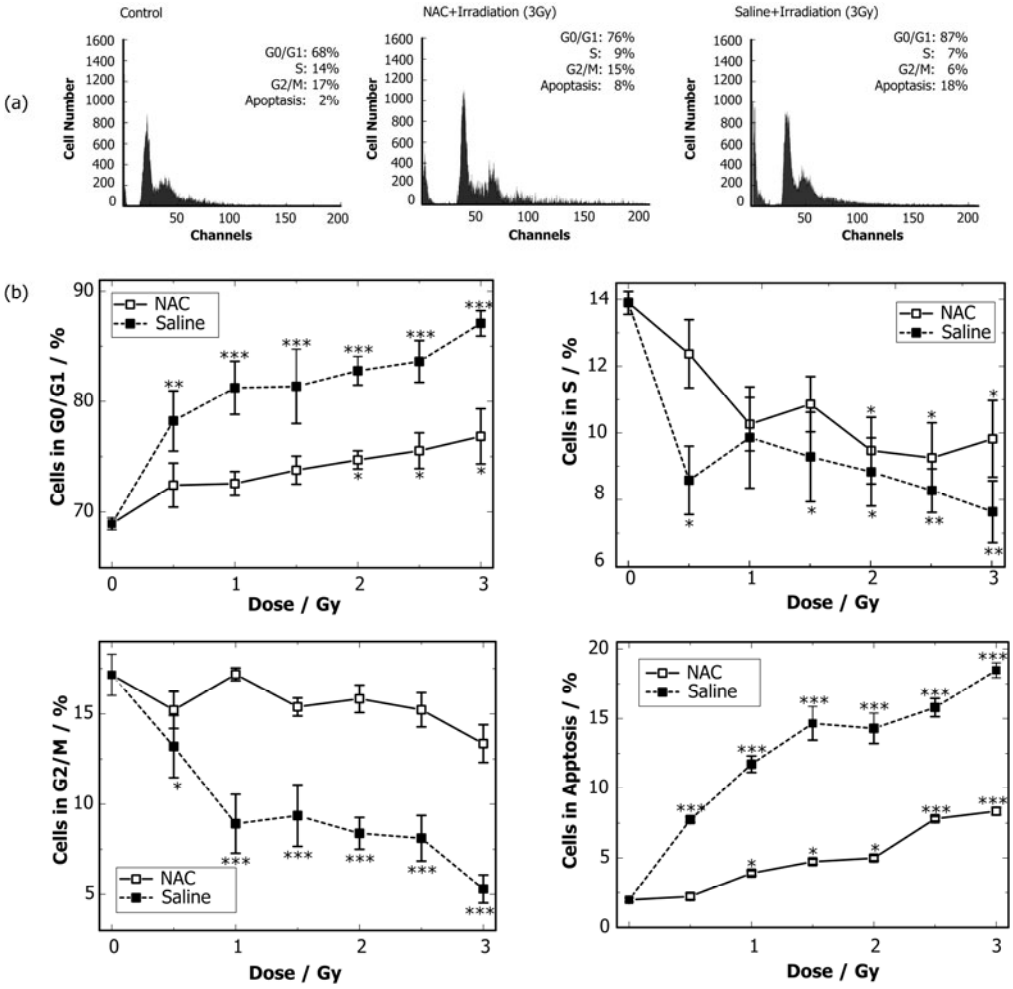

Fig.4 Effect of NAC on thymocytes cycle arrested and apoptosis. (a) Representative FACS images of thymocytes cycle and apoptosis. (b) Changes in the thymocytes cycle distribution and apoptosis percentage (**p*<0.05, ***p*<0.01, ****p*<0.001 *vs.* controls).

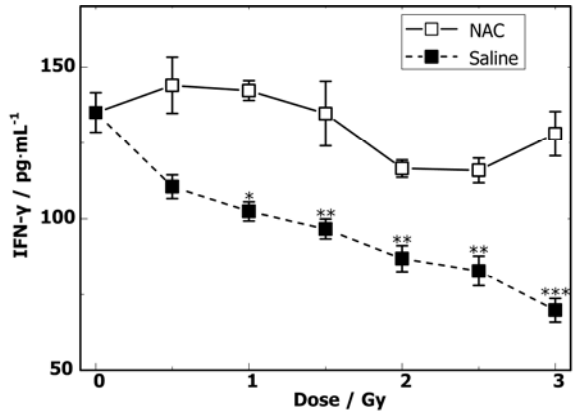

Fig.5 Effect of NAC on IFN-γ levels of mice exposure to carbon-ion irradiation (**p*<0.05,***p*<0.01,****p*<0.001 *vs.* controls).

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