# Toxicology Research

Accepted Manuscript






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# $^{56}$Fe irradiation-induced cognitive deficits through oxidative stress in mice

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**Running title:** $^{56}$Fe radiation-induced cognitive deficits in mice

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## Abstract

Aims: Rapid growth of manned space flight results in more concern about health risk and an urgent need of health assessment for space travel. Cosmic environment is complicated and full of radiation. Because of the high biology effective, heavy ion such as $^{56}$Fe ion is considered to be an important component of these lethal galactic rays. Since the importance of brain functions to astronauts, we explored the long-term effects and potential mechanisms of $^{56}$Fe ion radiation on mice brain containing the hippocampus.

Main methods: In our study, radiation doses were carried out with 0.5Gy, 1Gy or 2Gy. One month after whole-body $^{56}$Fe ion exposure, Morris water maze was performed to assess ability of spatial learning and memory. Histological study was used for pathology analysis of hippocampus. Alteration of oxidative stress was reflected by MDA and GSH and oxidative DNA damage marked by 8-OHdG was detected by biochemical and immunofluorescence methods.

Key findings: In our results, irradiated groups exhibited significant changes in behavioral performance and it also showed loose and edematous arrangement in pathological characters. Furthermore, whole brain levels of MDA, GSH and 8-OHdG increased in the irradiated groups. In addition, increased expression of 8-OHdG also can be detected by immunofluorescence in the hippocampus.

Significance: Our finding revealed a linkage between radiation-induced oxidative stress and behavioral deficits. This may suggest an underlying mechanism of brain tissue protection and risk assessment in manned space flight.

## Keywords

Radiation; Mice brain; Behavioral deficits; Oxidative damage, DNA damage

## Introduction

As the development of manned space flight, the duration and location of shuttle missions extend from past years. However, it is also increased the risks of central nervous system (CNS) damage which attributed to exposure to solar particles and cosmic rays. In general, these solar particles and cosmic rays are mainly consisted with high linear energy transfer (LET) ions such as photons and high (H) atomic number (Z) and high-energy (E) ions[1]. Although HZE particles are small part of cosmic rays, these highly eclectic charged ions contribute a dominant share of the effective dose and they also possess a strong ability of oxidative damage[2-4] which induce impairment of DNA and some other biological molecules[5]. In space, Fe ion is likely the most important component of cosmic rays, since the maximum contribution value of dose equivalent in the radiation spectrum[6]. For these reasons, Fe ion is ideal for ground-based research in space radiation. Studies have said there is a high uncertainty between 400% - 600% in radiation assessment for a Mars travel[7]. In large part, it is because the lack of knowledge about biological response to HZE particles. Therefore, understanding the acute and long-term effects of oxidative stress of response to HZE particles provides a theoretical basis to evaluate the risks of space travel and radiation protection. Here we use $^{56}$Fe ion beam to simulate HZE particles in cosmic environment to detect the underlying mechanisms of radiation-induced long-term effect on CNS.

It is known that whole-body exposure of mice to HZE particles may induce significant deficits on CNS [8-10]. Despite CNS is the most important system in the body and has been

fully researched, it remained uncertain how ionizing radiation exposure affected CNS. The underlying mechanisms are certain to be multifaceted and several mechanisms are considered to play a role in the radiation-induced deficits [11, 12], but oxidative stress may represent the direct and most important mechanistic explanation for it since studies shows that oxidative damage of nucleic acids, proteins, and lipids is directly correlated with neurodegeneration, aging, cardiovascular diseases and pathologies of some carcinoma [13-17]. Interactions between DNA and reactive oxygen species (ROS) induced by radiation in damaged cells leads to DNA strand-breaks and base modification which can be quantitatively estimated with 8-hydroxy-2'-deoxyguanosine (8-OHdG) produced by reaction of ROS on guanine ordinarily [18, 19] in animal organs and in human samples [20, 21].

In this study, we investigated the question of whether the brain can be adversely affected after 4 weeks by whole-body exposure with different doses (600 Mev/u, 0.5, 1 and 2Gy) of $^{56}$Fe ions irradiation. Experiments show that exposure to $^{56}$Fe beam resulted in significant impairment of cognitive performance. To further study causes of cognitive impairment, oxidative stress and DNA damage which may relate with cognitive deficits was evaluated by the levels of malondialdehyde (MDA), glutathione (GSH) and 8-OHdG. In addition, some other histological and biochemical experiments were also carried out. Finally, the studies imply these doses of $^{56}$Fe ion irradiation compromise cognitive performance through mechanisms involving changes of oxidative stress and oxidative DNA damage in brain tissue.

## Materials and methods

### Animals

SPF-class young adult male Kunming mice (6-8 weeks old, weighted 24-28g) obtained

from the Gansu University of Chinese Medicine (Gansu Province, China) were used for the study. The mice were maintained in a 12 h light/dark cycles and had free access to certified rodent diet and filtered water. All experiments of this study were carried out in accordance with the principles of Laboratory Animal Care and approved by Institutional Animal Care and Use Committee of Gansu University of Chinese Medicine (Permit Number: 2015-051). Furthermore, we also try to minimize animal suffering.

**Irradiation procedure**

Mice were divided in four groups, sham, and three-test (0.5Gy, 1Gy, 2Gy) groups (total32, n = 8 each group). The three-test groups were exposed to a single dose of total body $^{56}$Fe beam (600 Mev/u) irradiation without anesthesia at the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China) with a dose rate of approximately 1 Gy/min.

**Behavioral testing**

Morris water maze (MWM) apparatus (ZS Dichuang New Technological Development Limited Liability Company, Beijing, China) which consist of a circular metal pool (120 cm in diameter, 40 cm in height, filled to a depth of 21 cm with water at 21 ± 1 °C) and a video - capturing system were used to estimate the spatial learning and memory ability of mice.

MWM were virtually divided into four equivalent quadrants. 4 weeks after irradiation, mice were trained to locate a small round hidden platform which was submerged 2.0 cm below the surface of water in 2nd quadrant. There were 2 trials per session (15 min intertrial interval) and two sessions (3h apart) per day in five consecutive days. Each trial, the mice were moved to a new quadrant to locate the platform. The mice have 120s to find the hidden

platform by itself. After successful reaching, the mice need to remain on the platform for 10s to finish this trial. If the mice failed in 120s, this trial is also ended but the experimenter needs to place the mice on the hidden platform for 10s. After removal from the MWM, the mice were towel-dried and returned to their home cages.

At Day6 mice was placed in 4$^{th}$ quadrant to locate the hidden platform. The movement of mice in MWM was recorded for further studying. All tests were conducted in the morning and early afternoon (beginning at approximately 9:00 a.m. and 2:30 p.m., respectively).

**Tissue preparation**

24 hours after the last MWM experiment, mice were sacrificed by cervical dislocation. Brain tissue of each animal was dissected and quickly removed to cold physiological saline on ice for washing remaining blood. Then part of them placed and fixed in 4% paraformaldehyde (4g/100ml) with 0.01 mol/L phosphate buffer solution (PH 7.4) for analysis of hematoxylin and eosin staining (HE) and immunofluorescence. Remains tissues were soon stored at − 80°C for biochemical analysis.

**Biochemical analysis**

Brain tissues in one group, which stored at − 80°C, were mixed and homogenized in ice cold phosphate buffer solution (PH 7.4) by a mechanical homogenizer. Then the homogenate was centrifuged with 10 minutes at 2500rpm. After that, supernatant was used for analysis in levels of GSH, MDA and 8-OHdG with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (Shanghai Enzyme-linked Biotechnology, Shanghai, China). In these experiments, a Microplate Reader (infinite M200, TECAN, Switzerland) was used for detecting.

**Histological study**

Brain tissues which fixed in 4% paraformaldehyde were washed, dehydrated in ethanol with different concentration of 50% to 100%, cleared in xylene, and embedded in paraffin at 55°C for 3h. Paraffin blocks were cut with coronal sections by a microtome (Jung SM 2000R, Leica, Nussloch, Germany). Sections cut into 4-μm thickness were stained with hematoxylin and eosin (HE). Histopathological examinations were carried out under a microscope with camera and experienced observer to avoid any bias.

**Immunofluorescence analysis**

Immunofluorescence analysis was utilized to locate and determine of 8-OHdG in brain tissues sections. In brief, these sections were de-paraffinized, immerged in citrate solution for antigen retrieval with an environment of high temperature and pressure, treated with 0.2% Triton X-100 for 15 min at room temperature. Afterwards，sections were incubated with 1% bovine serum albumin (BSA) for 1 h and incubated with primary antibody to 8-OHdG (Beijing Biosynthesis Biotechnology, Beijing, China) (1:200 in PBS) overnight at 4˚C in PBS. Then paraffin sections were exposed to Alexa Fluor-488 goat anti-rabbit fluorochrome-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) with the concentration of 0.2% in Tris buffered saline (TBS) and maintained for 1h in the dark. Slides were washed three times in PBS and medium containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) was used for mounting in 5 minutes at room temperature. At last, cover slip was placed on the surface of slide. Expression and location of 8-OHdG was observed using a laser scanning confocal microscope with a digital camera (LSM700, Carl Zeiss).

### Statistical Analysis

There are at least three repetitions of each experiment. Data were presented as the mean ± standard error of the mean (SEM). Two tailed t-tests were carried out for single comparisons. Analysis of variance (ANOVA) and followed Student Neuman and Keuls (S-N-K) post-hoc comparisons was used for statistical comparison between different groups. A p-value of less than 0.05 was selected as a principle for a statistically significant difference.

## Results

### Morris water maze

MWM data of visible platform for 6 consecutive days are represented in Fig. 1. It can be observed that there was no aftermath of tests groups on swim velocity post-irradiation Fig.1A (0.5Gy, $P = 0.70$; 1Gy, $P = 0.25$; 2Gy, $P = 0.95$). Hence, escape latency can be used as measurement of performance. During trial sessions, all group animals showed experiment effect by the decrease of escape latency to reach the immersed platform (Fig. 1B). However, escape latencies of $^{56}$Fe ion exposed groups showed significantly raise ($P < 0.05$) compared to controls. The evidence can be found more conspicuous on day 2. On this day, mean escape latency of control, which decreased significantly compare to day 1, was found to be ~60s. But in the tests groups, it showed to be ~100s or ~110, similarity with day 1. Repeated measures of one way ANOVA analysis and post-hoc comparisons confirmed a significant prolong of escape latency between radiation groups and control in the last 5 days of MWM trials. In addition, our results indicated radiation groups showed a slight but significant abatement in percentage of time spent in the target quadrant (Fig. 1C) (63.61 ± 3.43, control; 49.25 ± 2.56, 0.5Gy; 47.74 ± 4.02, 1Gy; 44.29 ± 2.35, 2Gy) and repeated measures one way ANOVA with

post-hoc comparisons also affirmed that ($P < 0.05$). We also found animals in test groups are hard to reach the platform and an arbitrary swimming pattern can be seen (Fig. 1D). Furthermore, an interesting phenomenon can be seen during the experiment process. Some irradiated animals, especially in group of 2Gy, were just floating in the water and there was not any evident movement. Although these data were not included, that may indicate a mood disturbance.

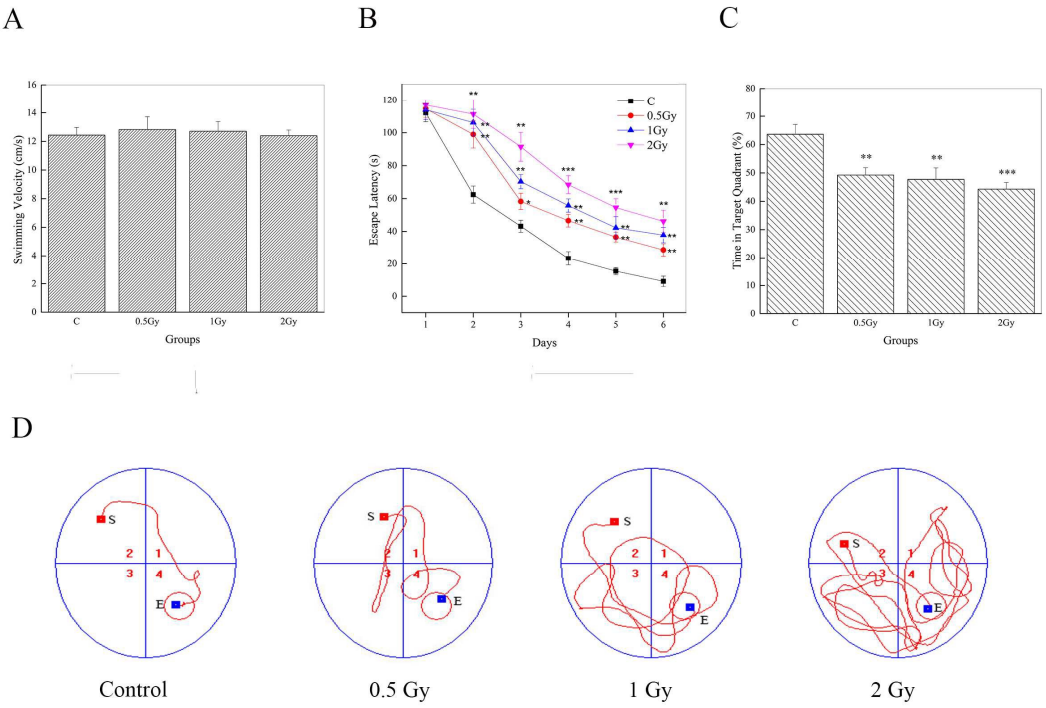


Fig 1, $^{56}$Fe ion radiation induced deficits of spatial learning and memory, one month after exposure. (A) All groups exhibited similar swimming speed in MWM tests. (B) Mice in irradiated groups showed significant increment of escape latency on days 2 – 6 compared to control group (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. control). (C) As comparison to control, percentage of time spent in target quadrant performed conspicuous decrease in irradiated groups (**$P < 0.01$ and ***$P < 0.001$ vs. control). (D) Representative tracing image

in tests trial on day 6.

**MDA and GSH activity analysis**

The levels of MDA and GSH in brain tissues were exhibited in Fig. 2. As comparison to controls (10.10 ± 0.55), our results showed a significant increase of MDA content (14.60 ± 0.55, $P < 0.01$, 1Gy; 18.78 ± 0.03, $P < 0.001$, 2Gy) in animals exposed to high dose (> 0.5Gy). However, a slight but not conspicuous increment can be seen in group of 0.5Gy (11.30 ± 0.11, $P = 0.10$) (Fig 2A). GSH concentration of mice in irradiated groups (12.50 ± 0.17, $P < 0.001$, 0.5Gy; 20.32 ± 0.72, $P < 0.001$, 1Gy; 16.65 ± 1.51, $P < 0.01$, 2Gy) were found to be significantly higher than control group (9.83 ± 0.36) (Fig. 2B). Interestingly, a slight abatement of GSH concentration was found in irradiated groups as comparison between 2Gy and 1Gy. MDA and GSH are important markers of oxidative stress. Their increment indicates a high degree of oxidative stress. Hence, the results showed an oxidative damage after $^{56}$Fe ion exposure.

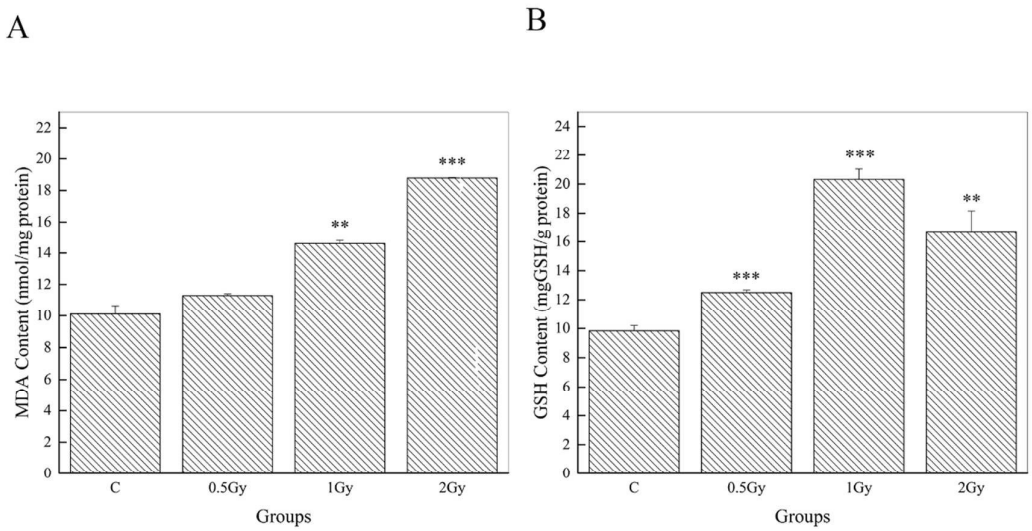

Fig 2, $^{56}$Fe ion radiation induced long term effects on oxidative stress. Graphs showing whole brain levels of MDA (A) and GSH (B) in groups of control, 0.5Gy, 1Gy and 2Gy (**P < 0.01 and ***P < 0.001 vs. control).

**Histopathological evaluations**

Results of MWM have indicated an impairment of cognitive and memory. To further detect radiation effects on it, our research focused on hippocampus and serial sections with HE staining was to be taken to histopathological analysis. In the group of control, neurons arranged tightly and displayed distinct cell structure with clear nucleus, nucleolus and edges (Fig. 3A). By contrast, hippocampus in groups of $^{56}$Fe ion exposure showed a conspicuous decrease in number of cells especially in edge of DG division (Fig. 3B - D). In addition, we also observed edema and loose arrangement occurred in the hippocampus of irradiated groups. These histopathological changes can be seen deteriorative as increasing dose of $^{56}$Fe ion exposure.

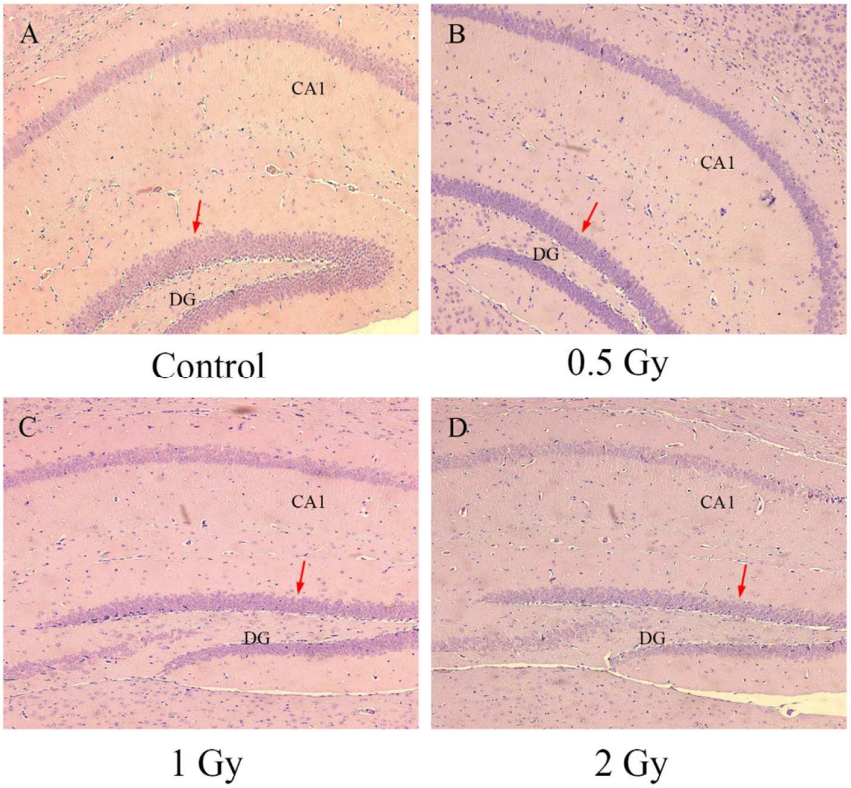


Fig 3, $^{56}$Fe ion radiation induced histopathological alteration on hippocampus. Groups of Control (A), 0.5Gy (B), 1Gy (C) and 2Gy (D) are displayed. Locations of cells decrease are showed with red arrow.

**DNA damage analysis**

As shown in Fig. 2, $^{56}$Fe ion significantly increased accumulated oxidative stress one month after exposure. Hence, we determined to detect whether radiation induced oxidative DNA damage in brain tissue. 8-OHdG which predominantly induced by free radical is a widely used biomarker of oxidative lesions [22]. Here we used a commercial measurement enzyme-linked immunosorbent assay (ELISA) kit to quantify the concentration of 8-OHdG in brain tissue and further detect its relative expression in hippocampus by immunofluorescence

analysis.

We observed the significant increases of 8-OHdG in both of irradiated groups (98.47 ± 3.35, $P < 0.01$, 0.5Gy; 138.04 ± 3.48, $P < 0.001$, 1Gy; 152.23 ± 1.2, $P < 0.001$, 2Gy) and nearly double of the level in group of 2Gy, as comparison to the control (79.02 ± 1.96) (Fig. 4A). Repeated measures of one way ANOVA and S-N-K post-hoc comparisons were used to affirm it.

As shown by immunofluorescence microscopy (Fig. 4B), we also found radiation induced noticeable DNA damage performed by 8-OHdG was located in CA1 area of hippocampus. In control group, nearly invisible immunoreactivities of 8-OHdG can be observed in CA1 division. However, the expression levels and intensity of 8-OHdG immunoreactivities was gradually increased as radiation dose escalation. These data indicate that radiation caused a serious aftermaths of DNA damage.

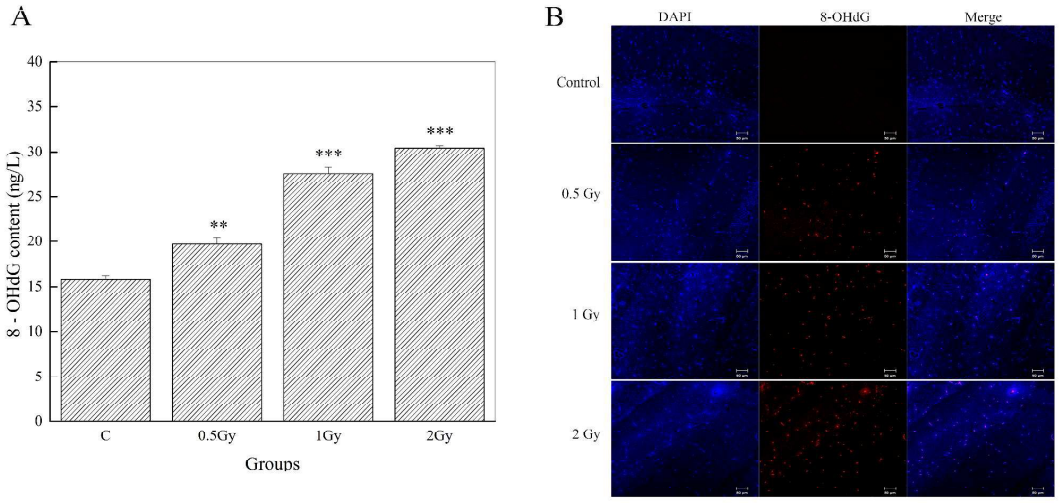


Fig 4, Graphs exhibit assessment of DNA oxidative damage induced by $^{56}Fe$ ion radiation. (A) Whole brain levels of 8-OHdG (**$P < 0.01$ and ***$P < 0.001$ vs. control). (B) Representative immunofluorescence analysis shows increase of 8-OHdG fluorescence (green) signal as

compared to control. Nuclei were stained with DAPI (blue).

## Discussion

Studies have shown HZE particles which are full of the cosmic environment induce various brain impairments in executive function, spatial learning and memory [23-25]. But most of articles focused on the acute-term and single dose effects of HZE particles radiation. As the increased duration of manned space flight, there is an urgent need to keep and even improve the astronaut's performance in cognition, memory and response. Hence, more attention should be paid to gradient-dose irradiation and long term effects of HZE particles. Then, our study was designed to detect long-term effects on cognitive impairment after exposure. $^{56}$Fe ion beam was used for simulating cosmic irradiation and gradient doses of 0.5Gy, 1Gy, 2Gy were carried out. Afterwards, we performed behavioral, biochemical, and histological analysis on brain tissue of mice.

Our results displayed conspicuous radiation effects on behavior which may associate with changes on physiology and cellular level. As a classic instrument of behavioral analysis, MWM was selected in our research for measuring spatial learning and memory retention, which depend on functions of intact hippocampus [26, 27].

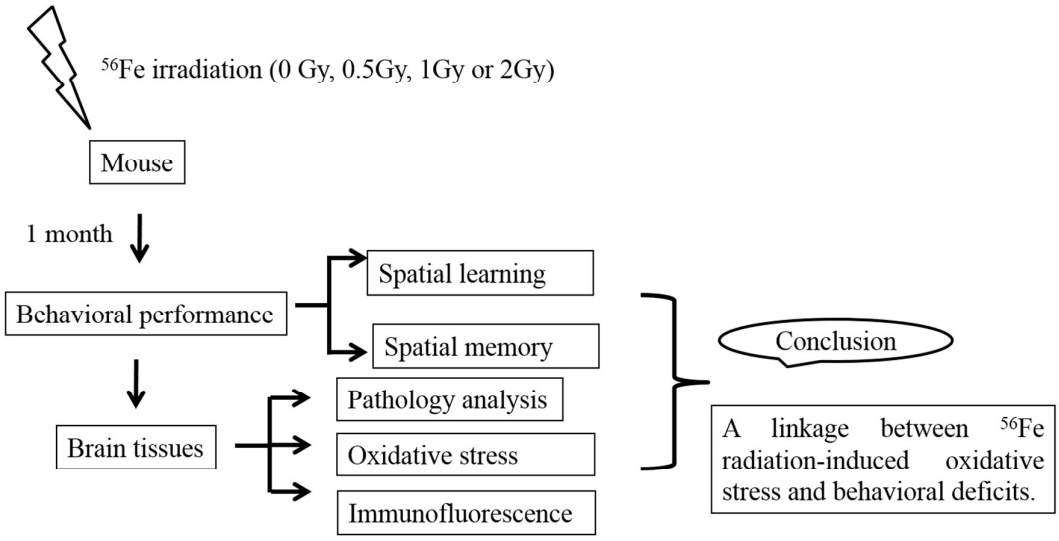


Fig 5, The diagrammatic representation of experimental design and conclusion.

In test of MWM, both groups showed experimental effect with decrease of escape latency during training trials. We found there were not significant changes in swimming velocity. That indicates radiation of $^{56}$Fe ion has no effect on motor ability of mice, which make escape latency and some other parameters of MWM available. Animals in irradiated groups showed significant prolong of escape latency (at least 3 times) in test session (6 day) as comparison to the controls. In training trials of day 2, a significant decrease of escape latency can be seen in control group while a slight decline of escape latency showed in the irradiated groups. Theoretically, mice in control group have intact ability of learning and memory. Therefore, they should spend more time in target quadrant to find it. Percentage of time spent in target quadrant proved this inference. It is significantly that mice in irradiated groups spent less time in target quadrant. Tracing images also verified that irradiated groups had a worse memory on location of platform. Based on these results, we suggested $^{56}$Fe ion radiation induces significant effects on spatial learning and memory and the aftermaths of radiation deteriorates as exposure dose increasing.

In addition, we also observed some freezing behavior in irradiated groups although it is a small probability event and our data did not cover it. Mice were found simply float and performed no movement in the water, especially in a high dose irradiation (2Gy) group. Studies shown this phenomenon may correlate with mood disturbance [28-30] . But few researches were carried on $^{56}$Fe ion beam and then this phenomenon may need to be further studied.

The behavioral effects induced by $^{56}$Fe ion radiation can be explained well by results of histopathological analysis. Brain tissues of mice in irradiated groups indicate a significant decrease and loose arrangement of cells, especially in the edge of the dentate gyrus (DG). The edge of DG is consisted with the granule cell layer (GCL) and subgranular zone (SGZ). These cells and structures are critical to brain functions. Studies shown granule cells in DG is critical to spatial memory formation [31, 32]. In fact adult-generated granule cells incorporate preferentially in process of spatial memory and loss of them brings about deficits of spatial memory [33]. The function of SGZ relate with neurogenesis and it is also reported there is a correlation between neurogenesis in the SGZ and spatial memory [34, 35]. On the other hand, the control group showed more cells and arranged tightly in this area. CA1 also played an important role in spatial learning. Other researches have shown CA1 is critical for processing temporal information of visible objects [36, 37]. However, edemas were found in this critical area as comparison to control. Then we suggest $^{56}$Fe ion made severe damage to hippocampus and these aberrant performances in pathology resulted in impairment of spatial memory.

To detect whether changes of oxidative stress were caused by $^{56}$Fe ion exposure, here we carried out biochemical analysis with biomarkers of antioxidation and lipid peroxidation. In

the process of radiation exposure, unpaired electrons cause various ROS which are potentially highly damaging to cell [3]. On the other hand, this process induces production of antioxidant and forms a dynamic balance in normal. Studies have shown there is a correlation of high oxidative stress, which induced by decrease of Non-enzymatic antioxidant GSH, and neurodegeneration [38]. The results showed there were significant changes in GSH after exposure. As compared with control, GSH level of irradiated groups indicated a conspicuous increase. But in groups of 2Gy, GSH level were shown a slight decrease in comparison with the group of 1Gy. We may suggest this phenomenon may be caused by over-consumption of GSH, which induced by higher oxidative stress of 2Gy radiation. In addition, it is also suggested GSH tolerance of $^{56}$Fe ion radiation (600 Mev/u) may in dose range of 1Gy to 2Gy. However, the underlying mechanisms need to be studied further. Researches have shown brain tissue has a high content of lipid [39] and MDA is one of an essential product of lipid peroxidation [40]. Hence, we determined to use MDA, a widely used marker of oxidative stress, as a parameter to assess oxidative damage in brain. In our results, MDA concentration is significant increase in tests group of 1Gy and 2Gy. However, there were no significant changes compared to control can be seen in group of 0.5Gy, although a slight increment on average existent. In this study, we affirmed $^{56}$Fe ion radiation negatively affected oxidative stress on brain tissue. But the effects of lipid peroxidation which induced by low dose radiation can be eliminated with personal antioxidant.

Oxidative lesions in DNA are thought to be the main reason of cell damage [41]. Oxidative damnification of guanine is a considerable reason to produce transversion of guanine for thymine [42, 43] and results accumulation of 8-OHdG subsequently in DNA [44]. Therefore,

nucleoside 8-OHdG can be utilized for estimating DNA damage[45]. Our ELISA analysis of 8-OHdG showed significant increase in irradiated groups. It is suggested $^{56}$Fe ion radiation has made grave injuries on DNA in brain cells and it can be detected even in one month later after exposure. We also suggested even if lipid peroxidation can be eliminated after one-month post-irradiation, but DNA damage cannot be cleared up in group of 0.5Gy.

CA1 is a subfield in hippocampus, which has been demonstrated critical with spatial learning and venerable to free radicles [36, 37, 46]. Hence, our work focused on detecting oxidative DNA damage in CA1 area of hippocampus. Results of immunofluorescence exhibited noteworthy expression of 8-OHdG in irradiated groups compared with control. Therefore, it indicates DNA oxidative damage induced by $^{56}$Fe ion radiation in CA1 of hippocampus is one of reasons on impairment of spatial learning and memory.

## Conclusions

In conclusion, we exhibited radiation induced increased expression of 8-OHdG in hippocampus with immunofluorescence after one-month exposure and also demonstrated $^{56}$Fe ion have a long-term effect on impairment of spatial learning and memory. These cognitive deficits were shown clearly to correlate with radiation-induced changes in pathology, increase of oxidative stress and oxidative DNA damage, which were detected in brain tissue and hippocampus of mice. In addition, we also found lipid peroxidation induced by low dose exposure can be eliminated in brain tissue, but DNA damage and behavioral deficits induced by changes of oxidative stress are still existent and the underlying mechanisms need to be further studied. Overall, our research proved correlation with long-term oxidative stress and behavioral deficits following exposure of the high-LET space

radiation ray. Our finding revealed a linkage between Fe ion radiation-induced oxidative stress and behavioral deficits and this may provide an experimental basis and ideas for further research of CNS protection and risk assessment.

**Acknowledgments**

This work was supported by grants from the Key Program of National Natural Science Foundation of China (U1432248), National Key Projects of Research and Development (2016YFC0904600), National Natural Science Foundation of China (No. 11305224; No. 11675234), and the Western Talent Program of Chinese Academy of Sciences (Y260230XB0).

**Conflicts of interest**

There are no conflicts of interest to declare

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