Radiation encephalopathy is the main complication of cranial radiotherapy. It can cause necrosis of brain tissue and cognitive dysfunction. Our previous work had proved that a natural antioxidant shikonin possessed protective effect on cerebral ischemic injury. Here we investigated the effects of shikonin on carbon ion beam induced radiation brain injury in mice. Pretreatment with shikonin significantly increased the SOD and CAT activities and the ratio of GSH/GSSG in mouse brain tissues compared with irradiated group (P<0.01), while obviously reduced the MDA and PCO contents and the ROS levels derived of the brain mitochondria. The shikonin also noticeably improved the spatial memory deficits caused by carbon ion beam irradiation. All results demonstrated that shikonin could improve the irradiated brain injury which might resulted from its modulation effects on the oxidative stress induced by the $^{12}$C$^{6+}$ ion beam.

Heavy-ions therapy possesses several advantages over conventional radiation therapy. However, therapeutic irradiation does not only kill tumor cells but also injures normal tissue. Radiation injury to living cells is, to a large extent, resulted from oxidative stress due to the excessive production of reactive oxygen species (ROS)\textsuperscript{[2].} It has been proved that high LET ($^{12}$C and $^{56}$Fe ions) irradiation disrupt neuronal systems and induce cognitive impairment. Our previous work has shown that a natural antioxidant shikonin could protect the mouse brain from the cerebral ischemic injury$^{[2]}$. Here, we investigated the effects of shikonin on the radiation brain injury induced by carbon ion irradiation.

A total of 100 SPF-class male adult Kunming mice (aged 6-7 weeks, weighted 18-22 g) were obtained from the Experimental Animal Center of Lanzhou University (Gansu Province, China). The mice were maintained in a 12 h light/dark cycle and had free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of National Institute of Pharmaceutical Education and Research. Mice were randomly assigned to the following five groups: control group, irradiated group and three different doses for shikonin-treated groups (shikonin, purity\textsuperscript{>98%} was purchased from Jiangxi Herb Technology Co., Ltd. Jiangxi, China). Each animal was fixed and given entire head uniform irradiation using a $^{12}$C$^{6+}$ ion beam (350 MeV/u primary energy, approximately 31.3 keV/µm LET in water) generated at the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China), at a dose rate of approximately 0.5 Gy/min. A 4.0 Gy dose of carbon ions was chosen in this experiment, since it is the most effective dose (based on our previous studies) and induces the least mortality in experimental mice. The control group did not receive any treatment. The irradiated group received entire head uniform irradiation with a carbon- ion beam (4.0 Gy) and the Shikonin groups received intraperitoneal injection of freshly prepared Shikonin at 10 mg/kg, 20 mg/kg, and 40 mg/kg, once a day for three days before the mice were exposed to the $^{12}$C$^{6+}$ beam.

In each group, 10 mice were killed by...
decapitation after 24 h irradiation, the brain tissue were minced and homogenized in ice-cold physiological saline. The antioxidative enzymes activities of superoxide dismutase (SOD) and catalase (CAT), levels of malondialdehyde (MDA), protein carbonyl content (PCO), reduced glutathione (GSH) and oxidized glutathione (GSSG) in brain tissues were measured by diagnostic reagent kit (supplied by Nanjing Jiancheng Bio-engineering, Nanjing, China) according to the specified method. ROS production in mouse brain mitochondria were detected by the membrane-permeable fluorescent probe 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma St. Louis, MO, USA)[3]. Mitochondria isolated from different groups (0.5 mg/mL) were incubated with 10 μmol/L DCFH-DA at 37 °C for 1 h, and the fluorescence intensity was measured at an excitation wavelength of 488 nm and emission wavelength of 525 nm. The ROS level was expressed as a percentage in fluorescence relative to irradiated control group. Moreover, 4 weeks after irradiation, each group was subjected for Morris Water Maze (MWM) testing to evaluate the spatial learning and memory ability.

All quantitative data were analyzed with SPSS 12.0 software (SPSS, USA) and expressed as mean±S.E.M. Statistical comparisons were performed with a Student’s t-test. Differences were considered significant at P<0.05.

Brain is considered especially sensitive to oxidative damage, and the ease with which neuronal membranes are peroxidized supports this notion. Brain tissue is enriched with the more easily peroxidizable fatty acids, consumes an inordinate fraction (20%) of the total oxygen consumption for its relatively small weight (2%), and is not particularly enriched in antioxidant defenses. It is known that lipid rich environments are more susceptible to free radical damage[4]. The present investigation shows that the activities of SOD and CAT decreased significantly after carbon ion irradiation, while the levels of MDA and protein carbonyl products increased inversely (shown in Table 1). The GSH/GSSG ratio is a quantitative biomarker of tissue oxidative status[5]. In comparison with the control group, the GSH/GSSG ratio sharply decreased after carbon ion irradiation. The results revealed that the carbon ion irradiation induced serious oxidative stress that over-consumed the endogenous antioxidative enzymes and antioxidants and resulted in the cell death. Our previous work had shown that an endogenous antioxidant melatonin inhibited the brain cell death induced by carbon ion beam irradiation[6]. Here, the natural antioxidant shikonin, was administrated to mice before the carbon ion exposure. The results indicated that the shikonin pretreatment significantly improved the carbon ions irradiation induced oxidative stress status in mouse brain tissue. Shikonin pretreatment prevented the decrease of the antioxidant enzymes activities and the GSH/GSSG ratio, and inhibited the increase of MDA and protein carbonyl formation in a dosage-dependent manner.

Mitochondria are the main intracellular source of reactive oxygen species[7], but they are also vulnerable to ROS’s attack[8]. Our recent work proved that the ROS derived from NADPH oxidase during the carbon ion irradiation injured the mitochondria and the impaired mitochondria became the executors of later cell death[9]. The fluorescent probe 2′, 7′-dichlorodihydrofluorescein was

**Table 1. Effects of Shikonin Pretreatment on the SOD, CAT Activities and the MDA and Protein Carbonyl (PCO) Levels in Mice Brain Tissue Irradiated by ¹²C⁺⁺ Ion Beam (±s, n=10)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Shikonin Treated (mg/kg)</th>
<th>¹²C⁺⁺ irradiation (Gy)</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>MDA Content (nmol/mg protein)</th>
<th>PCO (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group</td>
<td>-</td>
<td>-</td>
<td>90.83±5.92</td>
<td>62.83±3.99</td>
<td>101.61±5.73</td>
<td>10.45±4.04</td>
</tr>
<tr>
<td>Irradiation control</td>
<td>-</td>
<td>4.0</td>
<td>35.47±3.56</td>
<td>26.47±3.89</td>
<td>299.82±8.97</td>
<td>54.47±15.19</td>
</tr>
<tr>
<td>Shikonin treated groups</td>
<td>10</td>
<td>4.0</td>
<td>42.43±2.32</td>
<td>38.43±5.28</td>
<td>263.18±10.74</td>
<td>47.53±4.51</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4.0</td>
<td>53.12±3.31</td>
<td>43.15±2.33</td>
<td>207.21±9.22</td>
<td>36.25±5.82</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4.0</td>
<td>81.51±6.26</td>
<td>54.21±2.65</td>
<td>139.45±5.91</td>
<td>22.81±3.87</td>
</tr>
</tbody>
</table>

*Note. ***P<0.01, vs. control group; *P<0.05 vs. irradiation group. **P<0.01, vs. irradiation group.*
used to measure the level of ROS. As shown in Figure 1, the carbon ions irradiated brain mitochondria kept producing high level of ROS in comparison with normal control group. Pretreatment with shikonin significantly decreased brain mitochondrial ROS generation, which indicates that the neuroprotection conferred by shikonin is due to its anti-oxidative effect.

Functional evaluation is the most important way to identify the degree of radiation brain injury. The Morris Water Maze test is a classic experiment that evaluates brain cognitive function. Four weeks after the irradiation, four consecutive days of position navigation training, the escape latency decreased significantly as training times increased. Irradiated mice demonstrated cognitive impairment compared to the control group, as illustrated by their increased latencies to find the hidden platform. The escape latency was reduced in the irradiation group, but the degree of reduction was weak. During the spatial search test, residence time in the target quadrant and times through the virtual platform for $^{12}$C$^{6+}$ irradiation mice were significantly lower than those of that control group. The present findings corroborate previous reports suggesting the implication of ionizing radiation in cognitive dysfunction$^{[10]}$. Pretreatment different doses of shikonin reduced the escape latent period and improved the learning memory ability of irradiated mice in a dose-dependent manner (Figure 2).

Our results show that $^{12}$C$^{6+}$ ion beam irradiation can cause a decline in SOD and CAT activities, increase MDA and protein carbonyl content, and induce the GSH/GSSG ratio. This indicates that $^{12}$C$^{6+}$ heavy ion beam irradiation causes a series of oxidation in brain tissue, generating a large number of free radicals and induces brain tissue damage. Pretreatment with different doses of shikonin could act against oxidative stress and improve cognitive dysfunction.

The results suggest that when practicing cranial radiotherapy by $^{12}$C$^{6+}$ ion beam, it presents a risk of brain damage caused by radiation, and clinical treatment should attempt to avoid important regions of the brain that are associated with cognitive function. Our previous study showed that shikonin could protect mouse brain tissue against cerebral ischemia reperfusion injury through its antioxidant activity. Pretreatment with shikonin could protect brain tissue from radiation-induced brain injury. Furthermore, the antioxidant ability appears to be a basic and important mechanism of the neuroprotective effect of shikonin. The development of effective radio protectors is important for safe application of ionizing radiation therapy in medical practices.

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**Figure 1.** Effects of shikonin pretreatment on fluorescence intensity in mouse tissue caused by $^{12}$C$^{6+}$ ion beam irradiation ($\bar{x} \pm s, n=10$), **$P<0.01$ vs. normal control group; **$P<0.01$ vs. irradiation group. Ctr: normal control group; IR: carbon ion beam irradiated group, LS+IR: 10 mg/kg shikonin + 4 Gy irradiation; MS+IR: 20 mg/kg shikonin + 4 Gy irradiation; HR+IR: 40 mg/kg shikonin + 4 Gy irradiation.

**Figure 2.** Effects of shikonin pretreatment on spatial learning and memory in the spatial probe test of mice irradiated by a 350 MeV/u $^{12}$C$^{6+}$ ion beam ($\bar{x} \pm s, n=10$), **$P<0.01$, vs. control group; **$P<0.01$, vs. irradiation group. Ctr: control group; IR: irradiation group, LS+IR: 10 mg/kg shikonin + 4 Gy irradiation; MS+IR: 20 mg/kg shikonin + 4 Gy irradiation; HR+IR: 40 mg/kg shikonin + 4 Gy irradiation.
Protective effects of shikonin on radiation induced brain injury

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