

## Original Article

# Study of the radio-protective effect of cuttlefish ink on hemopoietic injury

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Irradiation leads to immunosuppression, hemopoiesis injury as well as sub-health of human being. The protective and therapeutic effects of cuttlefish ink on hemopoiesis in  $^{60}\text{Co}$   $\gamma$  radiated model mice were investigated. One hundred and twenty female ICR mice aged 6 weeks (20-24g) were randomly divided into five groups: the control group, the model group, and the low, medium, high dosage groups. The mice in different groups were orally administered normal solution (N.S.) or cuttlefish ink of different dosage daily for 40 days. Hemopoiesis impaired model was induced by  $^{60}\text{Co}$   $\gamma$  irradiating with lethal dose of 8.0 Gy. The number of bone marrow nucleated cells (BMNC), colony-forming unit in spleen (CFU-S), colony-forming unit of granulocyte and monocyte (CFU-GM), peripheral blood pictures and superoxide dismutase (SOD) activity in serum have been measured. Compared with model group, the decrease of BMNC, CFU-S, CFU-GM, peripheral leukocytes and SOD activity in serum in  $^{60}\text{Co}$   $\gamma$  irradiated mice of cuttlefish ink feeding groups were resisted significantly ( $p < 0.05$  or  $p < 0.01$ ). Moreover, the restoration of those indices was promoted significantly ( $p < 0.05$  or  $p < 0.01$ ). The cuttlefish ink showed no significant effect on peripheral erythrocytes, thrombocytes and hemoglobin. The results showed that cuttlefish ink had significant effects on granulopoiesis. The mechanism underlining these effects may be that the increase of antioxidant level in mice, the improvement of bone marrow haematopoietic microenvironment and the inducement of cellular factors promoted the proliferation and differentiation of CFU-S and CFU-GM and thus enhance the defensive system of organism.

**Key Words:** cuttlefish ink, irradiation injury, hemopoiesis, immune, superoxide dismutase

## Introduction

Irradiation is one of the extensive natural phenomena in the universe and our ambient environment. With the rapid development and the application of nuclear technology in agriculture, industry, medical and life science, people are increasingly exposed to artificial irradiation hazards. There are numerous health risks associated with irradiation such as immune organ atrophy and immunosuppression<sup>1</sup>, hemopoiesis injury and decrease of hematocytes<sup>2</sup>, as well as sub-health of human being. In addition, as an important therapeutic method for cancer<sup>3</sup>, radiotherapy has to be given up by many patients due to its strong side effects. One of the most important mechanisms of irradiation damage is peroxidation.<sup>4</sup> Reactive oxygen species such as super oxide anion radical and hydroxyl radical lead to cell death via inducing DNA oxidation, DNA strand breakage or base pair mismatch.<sup>5-6</sup> Although some effective chemical irradiation-protective agents have been found, the heavy adverse reactions prevented them from clinical application.<sup>7-8</sup> Therefore, it is one of our major concerns to find effective nutrition measurements, e.g., nontoxic anti-radiation functional foods and medicines from natural products.

Cuttlefish ink is a natural substance discharged by cuttlefish confronting enemies. It is mainly composed of melanin and protein-polysaccharide compound. Due to its hemostasia effects, cuttlefish ink is widely used in traditional Chinese medicine. The physical activities of cuttlefish ink,

such as anti-tumour, immunity promotion, and induction of many cytokines, have been widely researched in recent years.<sup>9</sup> Between two major components, melanin is an irregular polymer consisting of indole structure, which can resist free radicals chain reaction as the acceptor of free radical.<sup>10</sup> Previous study illustrated that cuttlefish ink can prevent acute irradiation syndrome<sup>11</sup> but the mechanism is not clear. However, little information on the hemopoietic function of cuttlefish ink was available. In this study, the effect of cuttlefish ink on hemopoietic function in hemopoiesis impaired model mice induced by  $^{60}\text{Co}$   $\gamma$  irradiation was investigated. The mechanism underlining such effect was proposed as well. The objective of this study is to deduce the effect of cuttlefish ink on humans via animal experiments, and develop a theoretical foundation for the clinical application of cuttlefish ink in health care and medicine.

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## Materials and methods

### Cuttlefish ink product

Fresh cuttlefish ink was hydrolyzed with 0.5% papain proteinase for 6 hours, then boiled for 10 minutes to denature the enzyme and washed with distilled water. The cuttlefish ink powder was obtained by vacuum drying at 60°C, stored at 4°C and suspended in distilled water for usage.

### Animals and design of the experiment

Healthy female ICR mice (weighing 22±2g, SPF, purchased from the Centre of Laboratory Animal of Qingdao) were divided into 5 groups randomly: control (neither irradiation nor cuttlefish ink), model (irradiation, no cuttlefish ink), low dosage (irradiation plus cuttlefish ink of 100mg/kg bw), medium dosage (irradiation plus cuttlefish ink of 300mg/kg bw) and high dosage group (irradiation plus cuttlefish ink of 900mg/kg bw), 24 mice in each group. Mice in control and model groups were administered N.S. as placebo. On Day 10, all mice except those in control group were placed in ventilated containers and were exposed to concentric revolving <sup>60</sup>Co treatment machine (FCC-7000, XINHUA iatrical instrument factory, China) at a rate of 50.8 cGy/min. The total dose of 8 Gy was given to the whole body. On Days 3, 10 and 30 after radiation, 8 mice in each group were sacrificed respectively and the number of bone marrow nucleated cells (BMNC), hemopoietic colony forming units in spleen (CFU-S), granulocyte-monocyte colony forming units (CFU-GM), the ratios of spleen/body weight and thymus/body weight were examined. Mean while, the dynamic change of peripheral blood picture in mice was observed on Days 0, 1, 3, 6, 8, 10, 14, 16, 21, 25 and 30 after irradiation, respectively.

### The ratios of spleen/body weight and thymus/body weight

The spleen and thymus of every mouse were taken out

and weighed immediately after sacrifice. The ratios of spleen/body weight and thymus/body weight were calculated at milligram per gram (mg/g).

### Bone marrow nucleated cells (BMNC) counting

Bone marrow cells were drawn from femoral bones with 3% acetic acid. Single-cell suspension was counted with haemocytometer.

### Spleen Colony Forming Unit (CFU-S) Assay<sup>12</sup>

Bone marrow cells from each donor were injected into tail veins of irradiated (8Gy) recipients (1×10<sup>5</sup> cells per mouse). After 9 days, recipient spleens were removed and fixed in Bouin's, and numbers of macroscopic colonies were counted.

### Lung conditioned culture medium of mice<sup>13</sup>

Two lung leaves of adult ICR mice were cut into pieces under the asepsis condition and cultured in 2ml RPMI-1640 with 10% horse serum in incubator (2711, QUEUE Systems, USA) at 37°C, 5% CO<sub>2</sub> and saturated humidity for 7 days. Then the supernatant was separated and stored at -20°C.

### Granulocyte-monocyte colony forming units (CFU-GM) Assay

CFU-GM was quantified in semisolid culture: 1×10<sup>5</sup> /ml bone marrow cells were cultured in RPMI-1640 containing 0.3% agar, 24% horse serum (Haoyang Biology Scientific Production Inc, Tianjin), 10% lung conditioned culture medium of mice and incubated at 37°C with 5% CO<sub>2</sub> for 7 days. Usually, cluster containing 50 or more cells was counted as a colony

### Peripheral blood picture

Blood was collected from tail veins of mice on different days following irradiation and the fluctuation of peripheral blood cells, including leukocytes, erythrocytes

**Table 1.** The effect of cuttlefish ink on the spleen and thymus index, the number of BMNC, CFU-S and CFU-GM in model mice after <sup>60</sup>Co γ irradiation (n=8,  $\bar{x} \pm s$ )

groups	Dose (mg.kg <sup>-1</sup> .d <sup>-1</sup> )	Spleen index (mg.g <sup>-1</sup> )	Thymus index (mg.g <sup>-1</sup> )	BMNC (×10 <sup>6</sup> .femur <sup>-1</sup> )	CFU-S (1×10 <sup>5</sup> cells) <sup>-1</sup>	CFU-GM (1×10 <sup>5</sup> cells) <sup>-1</sup>
Control	0	4.04±0.47	3.76±0.26	7.45±1.11	41.8±4.79	86.3±6.65
Preventive effect (D3 after irradiation)						
Model	0	1.17±0.13 <sup>a</sup>	0.56±0.14 <sup>a</sup>	1.18±0.06 <sup>a</sup>	19.8±3.77 <sup>a</sup>	47.7±4.11 <sup>a</sup>
Low dose	100	1.50±0.16 <sup>b</sup>	0.69±0.15	1.89±0.42 <sup>b</sup>	31.5±4.39 <sup>b</sup>	65.3±3.29 <sup>c</sup>
Medium dose	300	1.54±0.08 <sup>c</sup>	0.92±0.20 <sup>b</sup>	1.93±0.17 <sup>c</sup>	29.2±4.45 <sup>b</sup>	73.7±2.05 <sup>c</sup>
High dose	900	1.58±0.08 <sup>c</sup>	1.00±0.14 <sup>c</sup>	2.02±0.24 <sup>c</sup>	31.2±5.15 <sup>b</sup>	75.3±5.73 <sup>c</sup>
Therapeutic effect (D10 after irradiation)						
Model	0	0.95±0.16	0.74±0.18	0.82±0.21	16.8±1.79	46.0±6.97
Low dose	100	1.08±0.11	1.09±0.31	1.03±0.13	21.3±1.89 <sup>b</sup>	74.2±5.20 <sup>c</sup>
Medium dose	300	1.04±0.08	1.11±0.17 <sup>b</sup>	1.15±0.21 <sup>b</sup>	27.3±5.31 <sup>b</sup>	77.0±6.98 <sup>c</sup>
High dose	900	1.05±0.08	1.29±0.31 <sup>b</sup>	1.31±0.21 <sup>b</sup>	29.5±4.61 <sup>b</sup>	81.7±2.36 <sup>c</sup>
Therapeutic effect (D30 after irradiation)						
Model	0	3.91±0.27	1.38±0.35	1.89±0.05	28.2±2.74	54.7±3.68
Low dose	100	3.86±0.32	1.83±0.77	2.11±0.11	37.6±2.15 <sup>c</sup>	79.3±3.77 <sup>c</sup>
Medium dose	300	4.84±0.74	2.00±0.58 <sup>b</sup>	2.25±0.04 <sup>c</sup>	38.2±4.11 <sup>c</sup>	81.3±1.69 <sup>c</sup>
High dose	900	4.41±0.31	1.98±0.51 <sup>b</sup>	2.45±0.09 <sup>c</sup>	40.3±5.41 <sup>c</sup>	85.7±0.47 <sup>c</sup>

BMNC=bone marrow nucleated cells, CFU-S=colony-forming units in spleen, CFU-GM=colony-forming units of granulocyte and monocyte. <sup>a</sup>p<0.01, Compared with control; <sup>b</sup>p<0.05, <sup>c</sup>p<0.01, Compared with model

thrombocytes and hemoglobin levels were measured with an automatic hematocyte counter (SYSMAX F-820, Japan).

#### The SOD activity in serum

One hour after the last administration, blood was collected from the eyes and serum was separated. The SOD activity in serum was determined with the SOD reagent kit (catalogue No: 20020329, Nanjing Jiancheng Bioengineering Institute).

#### Statistical analyses

The results were expressed as the means±standard deviation. The statistical significance of the differences was evaluated with software Quattro Pro 9 and  $p < 0.05$  was considered as significant.

### Results

#### The ratio of spleen/body and thymus/body

The functions of spleen and thymus in mice were depressed by  $^{60}\text{Co}$   $\gamma$  irradiation (Table 1). On Day 3 after irradiation, the spleen and thymus indices of model group decreased by 71.0% and 85.1%, respectively. When mice were administered different dosage of cuttlefish ink for 9 days pre-irradiation, the spleen and thymus indices increased obviously in comparison with those of the model group. When mice were given cuttlefish ink after irradiation for 30 days, thymus index of the medium and high dosage groups recovered to 52.9% on average of the control, while the model group did merely to 36.7%. The results showed that cuttlefish ink had significant ( $p < 0.05$  or  $p < 0.01$ ) protective and therapeutic effects on immune organs in  $^{60}\text{Co}$   $\gamma$  injured mice.

#### Numbers of BMNC, CFU-S and CFU-GM

The hemopoietic function in mice was destroyed obviously by  $^{60}\text{Co}$   $\gamma$  irradiation (Table 1). Compared with the control, the numbers of BMNC, CFU-S, CFU-GM in mice of model group declined by 84.2%, 52.2% and 53.3%, respectively. However, when the mice were given

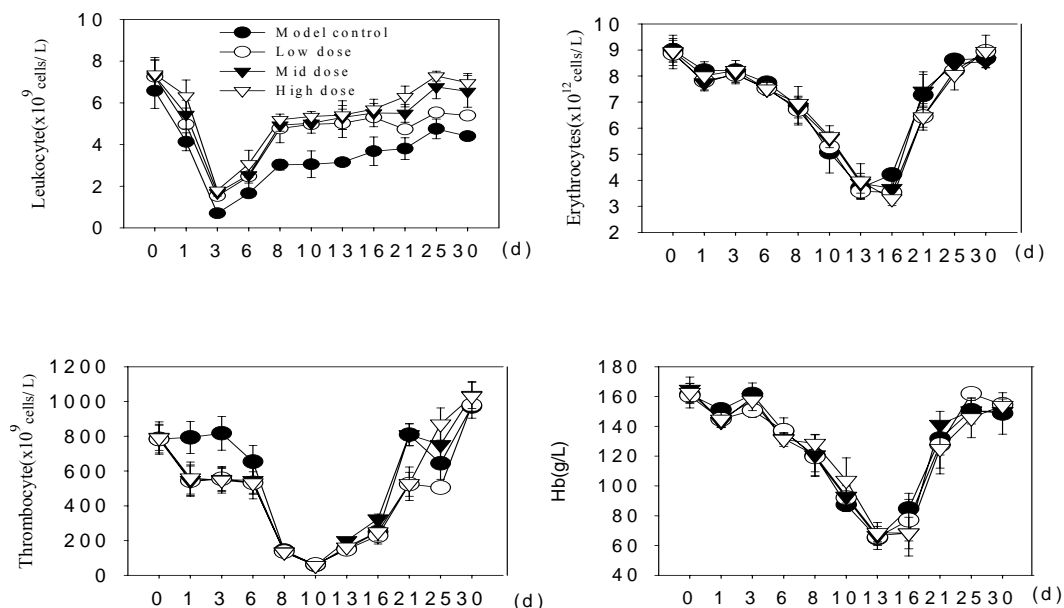
different dosages of cuttlefish ink for 9 days pre-irradiation, the numbers of BMNC, CFU-S, CFU-GM of cuttlefish ink feeding groups were 10.3%, 22.1% and 34.4% higher than those of model group, respectively. Up to Day 30 after irradiation, the indices mentioned above of cuttlefish ink feeding groups recovered to 30.5%, 96.6% and 99.2% of normal level, respectively, compared with model group did merely 25.4%, 67.6% and 59.9%. It illustrated that cuttlefish ink resisted the impairment and enhanced the recovery of hematoipoiesis effectively.

#### The fluctuation of peripheral blood picture

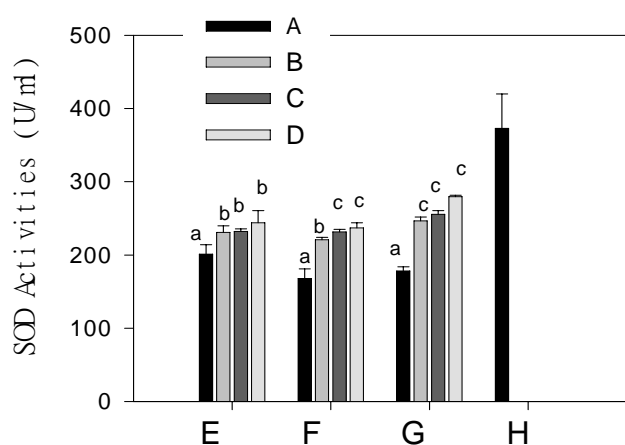
The numbers of leukocyte, erythrocyte, thrombocyte and hemoglobin changed markedly due to the hemopoietic damage in all irradiated groups. Among them, leukocytes were most sensitive to  $^{60}\text{Co}$   $\gamma$  irradiation (Figure 1). The leukocyte number declined rapidly at the beginning and then elevated gradually from Day 3 after irradiation. During post-irradiation period, the recovery of leukocytes of cuttlefish ink feeding groups was significantly rapider than those of model group ( $p < 0.01$ ). On Day 30 after irradiation, the number of leukocyte of cuttlefish ink feeding groups almost returned to the normal level, as comparison with 65.7% of the model group. The numbers of erythrocytes, thrombocytes and hemoglobin decreased slowly and reached to minimum values on Days 16, 10 and 13 after irradiation, respectively, then elevated gradually. But there was no statistical significance between dosage groups and model group.

#### The peroxidatic level

The SOD activity in serum was reduced by  $^{60}\text{Co}$   $\gamma$  irradiation ( $p < 0.01$ ). However, the SOD activity of cuttlefish ink feeding groups was markedly higher than that of model group during the post-irradiation period ( $p < 0.05$  or  $p < 0.01$ ) (Fig 2). On Day 30 after irradiation, the average SOD activity of cuttlefish ink feeding groups was 12.6% higher than that of the model group. The results suggested that cuttlefish ink had protective and therapeutic effects on the peroxidation induced by irradiation.



**Figure 1.** The effects of cuttlefish ink on dynamic change of peripheral blood picture in mice induced by  $^{60}\text{Co}$   $\gamma$  irradiation. Hb= hemoglobin.



**Figure 2.** The effects of cuttlefish ink on the level of peroxidation in mice induced by  $^{60}\text{Co}$   $\gamma$  irradiation. SOD=superoxide dismutase, A=Model, B=Low dose, C= Medium dose, D=High dose, E= D3 after irradiation, F= D10 after irradiation, G= D30 after irradiation, H=Control. <sup>a</sup>  $p < 0.01$ , Compared with control; <sup>b</sup>  $p < 0.05$ , <sup>c</sup>  $p < 0.01$ , Compared with model.

### Discussion

Hemopoiesis relates closely to the immunity of organism. BMNC denotes the hemopoietic function of marrow. Relative stability of peripheral blood cell number depends on the differentiation and proliferation of hemopoietic stem cells and progenitor cells. The interaction between hematopoietic cells and cell growth factors regulates the proliferation and differentiation of hematopoietic cells. It has been approved that cuttlefish ink can induce many kinds of cytokines such as IL-1<sup>14</sup> and colony stimulating factor (CSF)<sup>15</sup>. CSF stimulates the proliferation and differentiation of hemopoietic stem cell and many kinds of progenitor cells, increase the numbers of granulocyte, monocyte in blood and macrophage in tissue<sup>16-17</sup>. Recent research showed that IL-1 can promote the proliferation, differentiation and maturity of granulocytes while inhibit these processes of erythrocytes<sup>18</sup>. Our results showed that different dosages of cuttlefish ink could effectively resist the decrease of BMNC CFU-S, CFU-GM and peripheral leukocytes ( $p < 0.05$  or  $p < 0.01$ ) in  $^{60}\text{Co}$   $\gamma$  irradiated model mice. Moreover, the restoration of those indices mentioned above was promoted significantly ( $p < 0.05$  or  $p < 0.01$ ). However, there is no significant effect on peripheral erythrocytes, thrombocytes and hemoglobin. It suggested that cuttlefish ink could promote the proliferation of CFU-S and CFU-GM and induce them differentiating into granulocytes and monocytes, but had no effect on erythropoiesis.

Myelocytes and monocytes or macrophages derive from CFU-GM. There is GM-CSF receptor on the surface of CFU-GM membrane. Granulopoiesis and monopoiesis in organism are regulated mainly by GM-CSF during such derivation. CFU-GM semisolid cultured *in vitro* assay showed that the increase of CFU-GM was dose-dependent when exogenous GM-CSF presents. Our results illustrated that cuttlefish ink could stimulate stromal cells to secrete GM-CSF in hemopoietic microenvironment thereby promoting the proliferation, differentiation and maturity of CFU-GM in bone marrow. It has been proved that cuttlefish ink can activate T cells and B cells which in turn secrete large number of GM-CSF and other

cytokines, which finally promote the proliferation and differentiation of myelocyte progenitor cell<sup>19</sup>. Our findings evidenced further that the cuttlefish ink can promote the granulopoiesis in bone marrow.

Leukocyte plays a key role in the defensive system of organism. Both heterophile granulocytes (neutrophile in man) and monocytes have a marked capacity for ingesting small, discrete particles. The phagocytosis and digestion by the heterophile granulocytes is one of the means by which the host destroys bacteria, and the issue of some infections may depend upon the extent of phagocytosis. Moreover, activated monocyte and macrophage can synthesize and release various cytokines such as CSF, IL-1, IL-3, IL-6, tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon- $\alpha$  and interferon- $\beta$  (INF- $\alpha$ , INF- $\beta$ ), regulating cell growth and acting as a key factor in the inducement and regulation of immune responses. Cuttlefish ink could enhance non-specific immunity and specific immunity via promoting granulocytogenesis and monopoiesis in bone marrow.

The mechanism of hemopoietic injuries by irradiation may be the lipid peroxidation injury of stromal cells in bone marrow microenvironment<sup>20</sup>. Our results showed that cuttlefish ink could effectively resist the decrease of SOD activity in serum and promote its recovery. Melanin, as the main component of cuttlefish ink, has the ability of scavenging free radicals and antioxidation<sup>21</sup> thereby protecting hemotopoietic system from irradiation injury.

### Conclusion

Cuttlefish ink can promote the proliferation and the differentiation of granulocyte-monocyte progenitor cells, enhance non-specific immunity and specific immunity significantly. The mechanism may be that cuttlefish ink weakens the irradiation injury on hemopoietic microenvironment and hemopoietic cells via regulating immunological function, inducing GM-CSF and other cytokines and elevating SOD activity in mice. As a safe natural product, cuttlefish ink has potential clinical application in health care and medicine.

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