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The Yiqi and Yangyin Formula ameliorates injury to the hematopoietic system induced by total body irradiation

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ABSTRACT

In this study, we examined whether the Yiqi and Yangyin Formula (YYF), used in traditional Chinese medicine, could ameliorate damage to the hematopoietic system induced by total body irradiation (TBI). Treatment with 15 g/kg of YYF increased the survival rate of Institute of Cancer Research (ICR) mice exposed to 7.5 Gy TBI. Furthermore, YYF treatment increased the white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB) and hematocrit (HCT) counts in ICR mice exposed to 2 Gy or 4 Gy TBI. Treatment with YYF also increased the number of bone marrow cells, hematopoietic progenitor cells (HPCs), hematopoietic stem cells (HSCs) and the colony-forming ability of granulocyte–macrophage cells. YYF alleviated TBI-induced suppression of the differentiation ability of HPCs and HSCs and decreased the reactive oxygen species (ROS) levels in bone marrow mononuclear cells (BMMNCs), HPCs and HSCs from mice exposed to 2 Gy or 4 Gy TBI. Overall, our data suggest that YYF can ameliorate myelosuppression by reducing the intracellular ROS levels in hematopoietic cells after TBI at doses of 2 Gy and 4 Gy.

KEYWORDS: Yiqi and Yangyin Formula, ionizing irradiation, hematopoietic system, reactive oxygen species

INTRODUCTION

Total body irradiation (TBI) may induce cell and tissue injury through direct ionization effects and through the generation of free radicals caused by water radiolysis [1]. Doses of TBI of between 1 and 10 Gy delivered to rodents cause acute hematopoietic syndrome, residual bone marrow (BM) injury and hematopoietic stem cell (HSC) senescence [2, 3], suggesting that the hematopoietic system is highly radiosensitive. Therefore, there is a need for effective treatments to ameliorate radiation-induced injury. Amifostine is a radioprotective drug that has been approved by the US Food and Drug Administration (FDA) to protect against radiation therapy [4]. However, treatment with amifostine causes severe side effects, including hypotension, fatigue and somnolence. These side effects ultimately led to the termination of treatment in 15–20% of the patients in the clinical study [5, 6]. Therefore, it is necessary to explore alternative treatment options for irradiation protection with better efficacy and less serious side effects.

In October 2015, Youyou Tu won the Nobel Prize in Physiology or Medicine for her contribution to the discovery of the antimalarial drug artemisinin [7, 8]. Because artemisinin was isolated from a plant used in traditional Chinese Medicine (TCM), it is thought that other compounds used in TCM may also have potential uses in the clinical treatment of disease [9–12]. The Yiqi and Yangyin Formula (YYF) is used in TCM and contains the following compounds: *Astragalus* root, ginseng, glossy privet fruit, *Eclipta alba*, Chinese *Angelica*, Bighead *Atractylodes* rhizome, *Wolfiporia extensa*, and Radix *Glycyrrhizae* Preparata. YYF is widely used in the clinical setting to improve immunologic function and to promote overall health [13]. In

addition, YYF may reduce the resistance of leukemia stem cells to drugs in the context of minimal residual leukemia [14, 15]. These results suggest that YYF can promote hematopoietic recovery in cancer patients. In the present study, we examined the protective effects of YYF on TBI-induced hematopoietic system injury and discuss the possible mechanisms underlying these effects. Our findings show that YYF may mitigate TBI-induced myelosuppression by reducing the reactive oxygen species (ROS) levels in hematopoietic cells.

MATERIALS AND METHODS

Preparation of drugs

The YYF compounds were provided by Professor Zhexin Shi, who is an expert on pharmacological identification from the School of Tianjin University of Traditional Medicine and a doctor at the First Affiliated Hospital of Tianjin University of Traditional Medicine. The composition of YFF is specified in Table 1. The compounds were dissolved in distilled water to a concentration of 1 g/ml, and the solution was sterilized in an Arnold sterilizer.

Mice

Male Institute of Cancer Research (ICR) mice were purchased from Vital River (Beijing, China). The mice were bred at a certified animal care facility at the Institute of Radiation Medicine of Peking Union Medical College (IRM-PUMC); 6–8-week-old mice were used in all of the experiments. All of the animal experiments in our study were approved by the Institutional Animal Care and Use Committee of IRM (No. 1204).

Total body irradiation and YYF administration

Mice that were used in the survival experiments were randomly assigned into the following five groups: control, TBI, TBI + low-dose YYF (5 g/kg), TBI + middle-dose YYF (15 g/kg) and TBI + high-dose YYF (45 g/kg). Individual mice in the TBI + YYF groups were administered YYF by oral gavage every day for 8 days following TBI. All the mice receiving TBI for the survival experiments were exposed to 7.5 Gy using ^{137}Cs γ-rays (Cammacell-40, Atomic Energy, Mississauga, ON, Canada) at a rate of 1.0 Gy/min.

Table 1. Composition of Yiqi and Yangyin Formula (YYF)

Medicinal plant	Amount (g)
Astragalus root	30
Ginseng	10
Glossy privet fruit	15
Eclipta alba	15
Chinese *Angelica*	10
Bighead *Atractylodes* rhizome	10
Wolfiporia extensa	10
Radix *Glycyrrhizae* Preparata	10

For the remaining experiments, mice were divided into the following four groups: control, YYF, TBI (2 Gy or 4 Gy), and TBI (2 Gy or 4 Gy) + YYF. Individual mice in the YYF group and the TBI (2 Gy or 4 Gy) + YYF group received a dose of 15 g/kg YYF administered by oral gavage every day for 8 days following 2 Gy or 4 Gy TBI. All the mice receiving TBI were exposed to a dose of 2 Gy or 4 Gy using ^{137}Cs γ-rays at a rate of 1.0 Gy/min. Control mice were sham-irradiated.

Peripheral blood cell and bone marrow mononuclear cell counts

Nine days after receiving a dose of 2 Gy TBI, or 15 days after receiving a dose of 4 Gy TBI, blood was collected from the mice via the orbital sinus using a micropipette coated with an anticoagulant. The following measurements were collected: white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB) and hematocrit (HCT) counts. Bone marrow mononuclear cells (BMMNCs) were isolated and collected as previously described [16, 17], and BMMNCs were counted using a Celltac E hemocytometer (Nihon Kohden, Japan) and expressed as 10^6 cells/femur.

Flow cytometric analysis

BM cells were isolated by flushing both tibias and femurs with sterile phosphate buffer saline (PBS). The cells were filtered and counted prior to staining with antibodies. For the analysis of HSCs and hematopoietic progenitor cells (HPCs), 5×10^6 BM cells were stained with biotin-conjugated antibodies specific for murine CD4, CD8, CD11b, CD45R/B220, Ter-119 and Gr-1 and then stained with streptavidin–PerCP, anti-Sca-1–PE and anti-c-kit–APC. The HPCs ($Lin^-c\text{-}kit^+Sca\text{-}1^-$) and HSCs ($Lin^-c\text{-}kit^+Sca\text{-}1^+$) were analyzed using a BD Accuri C6 Flow Cytometer. For the detection of ROS, 5×10^6 BM cells were stained with the HPC and HSC markers described above and then incubated with 2,7-dichlorodihydrofluorescein diacetate (DCFDA, 10 μM) for 30 min in a 37°C water bath. The intracellular ROS levels in BMMNCs, HPCs and HSCs were measured using the mean fluorescence intensity (MFI) of DCFDA [18].

Colony-forming unit–granulocyte-macrophage (CFU-GM) assay

The colony-forming unit–granulocyte-macrophage (CFU-GM) assay was performed by culturing BM cells in MethoCult GF M3534 methylcellulose medium (Stem Cell Technologies, Vancouver, BC, and Canada). CFU-GM colonies with more than 30 cells were tallied, as per the manufacturer's instructions. The results are expressed as the number of CFU-GMs per 10^5cells [19, 20].

Statistical analysis

All the data were analyzed using GraphPad Prism5 software with Welch's *t*-test (significantly different was defined as $P < 0.05$). Survival analyses were performed using the log-rank test.

RESULTS

YYF increased the survival of ICR mice after 7.5 Gy TBI

In this study, mice were exposed to a lethal dose of TBI (7.5 Gy) to evaluate the protective effects of YYF on survival *in vivo*. As shown

in Fig. 1, 30% of the mice in the TBI group survived for 30 days. By contrast, in the mice treated with YYF, the survival rates were 40%, 80% and 50% in the low-dose, middle-dose and high-dose YFF groups, respectively. The survival rate in the middle-dose YYF group was significantly higher than that in the TBI group, suggesting that YYF may effectively protect mice from irradiation injury.

YYF attenuates myelosuppression in ICR mice after TBI

To examine the protective effect of YYF, the mice were exposed to 2 Gy or 4 Gy TBI, doses likely to induce damage to the hematopoietic system. As shown in Tables 2 and 3, the WBC, RBC, HGB and HCT counts decreased in the TBI group ($P < 0.05$) compared with the respective counts in the control group. Treatment with YYF alleviated the TBI-induced damage to the peripheral blood cells, increasing the WBC, RBC, HGB and HCT counts. These results show that YYF may attenuate the myelosuppression induced by 2 Gy or 4 Gy TBI.

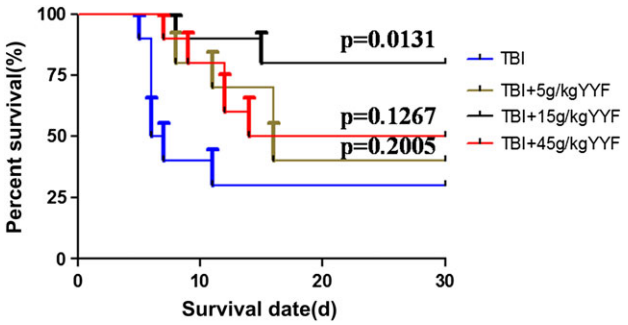

Fig. 1. YYF administration increased survival *in vivo*. Log-rank test of animal survival following exposure to a lethal dose of TBI. $n = 10$ mice per group.

YYF increased the number of bone marrow cells after TBI

TBI may induce hematopoietic system damage, causing a decrease in both the numbers and the self-renewal ability of HPCs and HSCs. As shown in Fig. 2, the numbers of HPCs, HSCs and BMMNCs per femur decreased significantly after 2 Gy or 4 Gy TBI ($P < 0.05$) compared with the numbers in the control group. By contrast, in the mice that were exposed to 2 Gy or 4 Gy TBI and treated with YYF, the numbers of BMCs, HPCs and HSCs recovered ($P < 0.05$).

YYF increased bone marrow cell self-renewal ability after TBI

The self-renewal ability of BM cells was assessed in a CFU-GM assay. Compared with the control group, the number of CFU-GM cells per 10^5 BM cells in the TBI group was significantly reduced ($P < 0.01$). However, the reduction of CFU-GM frequency was attenuated upon treatment with 15 g/kg YYF after TBI ($P < 0.01$; Fig. 3). All these findings suggest that YYF may increase both the number of HPCs and HSCs and the self-renewal ability of the HPCs after TBI.

YYF decreased the ROS levels in hematopoietic cells following TBI

TBI may induce the production of free radicals, contributing to cell and tissue damage. Compared with the control group levels, the ROS levels in BMMNCs, HPCs and HSCs increased nearly 2-fold in the TBI group ($P < 0.05$). When mice were treated with 15 g/kg YYF after TBI, the ROS levels significantly decreased ($P < 0.05$;

Table 2. Variation in peripheral blood parameters of mice treated with YYF as exposed to 2 Gy TBI

Group	WBC ($\times 10^3$/mm^3)	RBC ($\times 10^3$/mm^3)	HGB (g/dl)	HCT (%)	PLT ($\times 10^3$/mm^3)
Ctr	8.6 ± 1.5	9000 ± 300	14.42 ± 0.28	35.0 ± 1.0	494.8 ± 51.1
YYF	8.2 ± 1.5	8500 ± 700	13.78 ± 0.87	32.8 ± 2.0	562.6 ± 87.3
2 Gy	3.8 ± 0.4*	7600 ± 300*	12.50 ± 0.65*	30.2 ± 1.6*	306.8 ± 41.8*
2 Gy + YYF	4.9 ± 0.4**	8700 ± 600**	14.03 ± 0.46**	33.6 ± 0.6**	296.3 ± 89.2

Mice were sham-irradiated as a control group (Ctr) or irradiated with 2.0 Gy total body irradiation (TBI). Blood was collected and cells were counted after the mice were euthanized 9 days after receiving 2 Gy TBI. The data are expressed as the mean ± SD ($n = 5$ for each group). YYF = Yiqi and Yangyin Formula, WBC = white blood cell, HGB = hemoglobin, HCT = hematocrit, PLT = platelet. *$P < 0.01$ (vs the control group); **$P < 0.05$ (vs the 2 Gy group).

Table 3. Variation in peripheral blood parameters of mice treated with YYF as exposed to 4 Gy TBI

Group	WBC ($\times 10^3$/mm^3)	RBC ($\times 10^3$/mm^3)	HGB (g/dl)	HCT (%)	PLT ($\times 10^3$/mm^3)
Ctr	7.8 ± 1.5	10700 ± 7000	15.96 ± 0.78	36.6 ± 2.2	542.3 ± 112.7
YYF	7.9 ± 2.6	11200 ± 2000	15.96 ± 0.53	37.8 ± 1.3	509.6 ± 172.3
4 Gy	1.9 ± 0.4*	7800 ± 3000*	12.76 ± 0.32*	30.9 ± 0.0*	203.0 ± 99.5*
4 Gy + YYF	2.5 ± 0.2**	9200 ± 4000***	15.50 ± 0.22***	33.4 ± 1.0***	217.4 ± 67.3

Mice were sham-irradiated as control (Ctr) or irradiated with 4.0 Gy total body irradiation (TBI). Blood was collected and cells were counted after the mice were euthanized 9 days after receiving 4 Gy TBI. The data are expressed as the mean ± SD ($n = 5$ for each group). YYF = Yiqi and Yangyin Formula, WBC = white blood cell, HGB = hemoglobin, HCT = hematocrit, PLT = platelet. *$P < 0.05$ (vs the control group); **$P < 0.01$ (vs the 2 Gy group); ***$P < 0.05$ (vs the 2 Gy group).

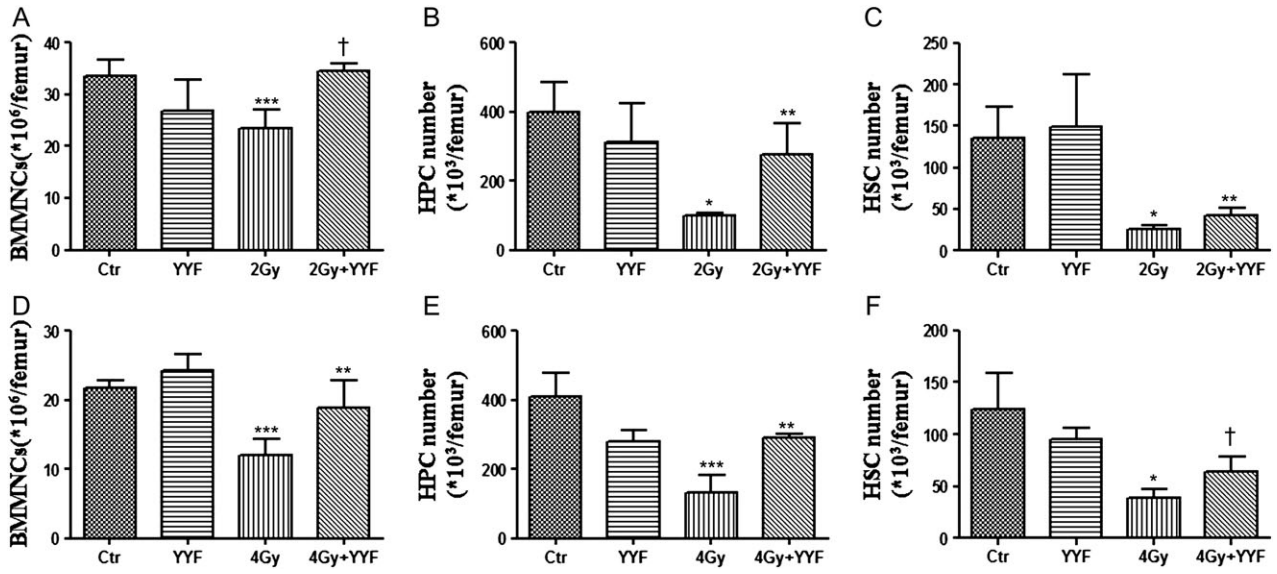

Fig. 2. YYF increased the number of BMMNCs after TBI. (A) Bar graph showing the number of BMMNCs per femur in mice after receiving 2 Gy TBI; (B) bar graph showing the number of hematopoietic progenitor cells (HPCs), lineage-negative, c-kit–positive and Sca-1–negative cells per femur in mice after receiving 2 Gy TBI; (C) bar graph showing the number of hematopoietic stem cells (HSCs), lineage-negative, c-kit–positive and Sca-1–positive cells per femur in mice after receiving 2 Gy TBI; (D) bar graph showing the number of BMMNCs per femur in mice after receiving 4 Gy TBI; (E) bar graph showing the number of HPCs, lineage-negative, c-kit–positive and Sca-1–negative cells per femur in mice after receiving 4 Gy TBI; and (F) bar graph showing the number of HSCs, lineage-negative, c-kit–positive and Sca-1–positive cells per femur in mice after receiving 4 Gy TBI. The data are expressed as the means ± SD ($n = 5$ for each group). *$P < 0.01$ vs the control group; **$P < 0.05$ vs the TBI (2 Gy or 4 Gy) group; ***$P < 0.05$ vs the control group; and $^{\dagger}P < 0.01$ vs the TBI (2 Gy or 4 Gy) group.

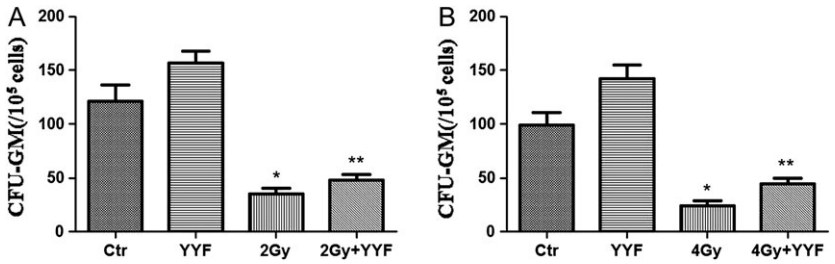

Fig. 3. YYF increased the colony-forming ability after TBI. (A) Bar graph showing the number of CFU-GMs formed by 10^5 BMMNCs in mice after receiving 2 Gy TBI; and (B) bar graph showing the number of CFU-GMs formed by 10^5 BMMNCs in mice after receiving 4 Gy TBI. The data are expressed as the mean ± SD ($n = 5$ for each group). *$P < 0.01$ vs the control group; **$P < 0.01$ vs the TBI (2 Gy or 4 Gy) group.

Fig. 4). Overall, these data suggest that YYF may scavenge free radicals in the BMMNCs to alleviate TBI-induced damage.

DISCUSSION

The hematopoietic system is sensitive to environmental stressors, such as benzene and its derivatives, ionizing radiation, and air pollution [1, 21, 22]. All these environmental health hazards may cause damage to the hematopoietic system. Ionizing radiation is encountered frequently during a person's lifetime, and the side effects are difficult to avoid [23, 24]. Oral mucositis [25] and radiation enteritis [26] are both common side effects in patients with head and neck cancer or pelvic tumors (respectively) who have undergone radiation therapy. In addition, it is difficult to protect the pericarcinous tissues from radiation injury [27, 28]. Previous reports have suggested that compounds used in Chinese herbal medicine may be able to mitigate TBI-induced damage in the brain [29], myocardium [30], esophagus [31] and BM [32, 33] of irradiated mice or rats. It has been shown that YYF can promote hematopoietic recovery [13, 15]. In the present study, we demonstrated that YYF had a protective effect on TBI-induced hematopoietic system damage.

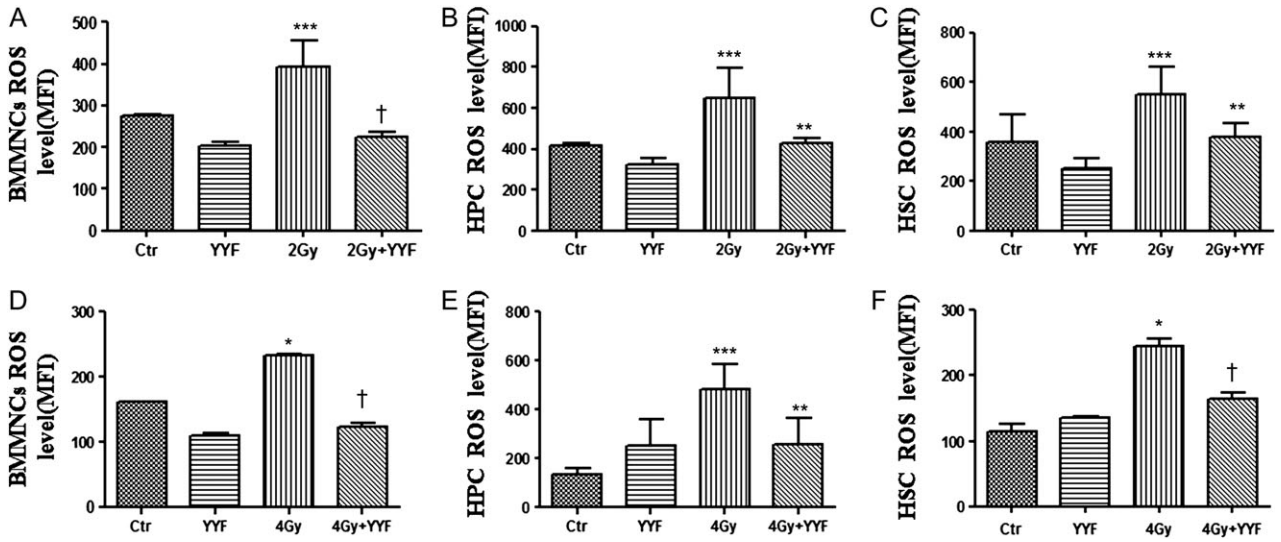

Fig. 4. YYF decreased the ROS levels in BMMNCs from irradiated mice. (A) Bar graph showing the levels of intracellular ROS in BMMNCs after 2 Gy total body irradiation (TBI); (B) bar graph showing the levels of intracellular ROS in hematopoietic progenitor cells (HPCs) after 2 Gy TBI; (C) bar graph showing the levels of intracellular ROS in hematopoietic stem cells (HSCs) after 2 Gy TBI; (D) bar graph showing the levels of intracellular ROS in BMMNCs after 4 Gy TBI; (E) bar graph showing the levels of intracellular ROS in HPCs after 4 Gy TBI; and (F) bar graph showing the levels of intracellular ROS in HSCs after 2 Gy TBI. The data are expressed as the mean ± SD ($n = 5$ for each group). *$P < 0.01$ vs the control group; **$P < 0.05$ vs the TBI (2 Gy or 4 Gy) group; ***$P < 0.05$ vs the control group; and $^{\dagger}P < 0.01$ vs the TBI (2 Gy or 4 Gy) group.

We first evaluated the survival rate of ICR mice after TBI. Thirty days after the mice were exposed to a lethal dose of TBI, 30% of the mice in the TBI group were alive, whereas the survival rate increased to 80% in mice treated with 15 g/kg YYF. However, the survival rates were similar in the TBI group and both the low-dose YYF (5 g/kg) and high-dose YFF (45 g/kg) groups (Fig. 1). These data suggest that YYF can protect against TBI-induced damage in ICR mice, but that the dose of YYF may be important. Low-dose YYF was unable to protect the mice from TBI-induced injury, and the high dose of YYF may have led to adverse side effects in the mice. These results are similar to those reported in many previously published studies [33–35]. TBI may also induce myelosuppression, reduced self-renewal ability and differentiation in HPCs and HSCs, as well as decreased numbers of BMMNCs. Following TBI (at a dose of either 2 Gy or 4 Gy) in combination with 15 g/kg YYF, the WBC, RBC, HGB and HCT counts increased significantly compared with the counts in the TBI group (Table 2). These data suggest that YYF may alleviate the TBI-induced suppression of differentiation ability in HPCs and HSCs. Moreover, YYF may alleviate the TBI-induced damage to BMMNCs, since the CFU-GM increased (Figs 2 and 3). It has been previously shown that ROS plays an important role in mediating BM cell injury [36–38]. Here, we found that YYF may decrease the ROS levels of BMMNCs, HPCs and HSCs in mice that have received 2 Gy or 4 Gy TBI (Fig. 4). Overall, our data suggest that YYF may alleviate TBI-induced myelosuppression by reducing the ROS levels.

Our results show that compounds used in TCM may have a protective effect on TBI-induced hematopoietic system injury. These results are consistent with previous findings [32, 33] and suggest that YFF or other TCM may be candidate agents for radioprotection. As for many other Chinese herbal compounds, little is known about the adverse effects or toxicity of YYF. However, during the course of our study, treatment with 45 g/kg YYF did not increase the survival rate of mice receiving 7.5 Gy TBI, suggesting that YYF at a high dosage may result in adverse side effects or toxicity. We investigated the reported adverse effects of each of the individual components in YFF and found that a very high dose of some of these components may result in adverse side effects or toxicity. For example, people who take a very high dose of *Astragalus* may develop insomnia, hypertension and dizziness. Additionally, excessive intake of ginseng may induce an acute toxic reaction, with symptoms such as fever, headache, and shortness of breath; a high dose of Chinese *Angelica* may give rise to symptoms such as feeling sleepy and tired; and excessive use of Radix *Glycyrrhizae* Preparata may lead to hypertension, headache, muscle injury and other side effects. However, the adverse effects of these Chinese herbal compounds may be due to improper use. YYF has been used widely, with few adverse effects reported in the clinical setting. In addition, a dose of 15 g/kg YFF in mice is a safe and effective dose in humans.

However, it is difficult to determine which TCM components are effective; thus, more detailed research is needed. We demonstrated that YYF conferred protection against TBI-induced myelosuppression, and we also found that YYF played a limited role in radioprotection. The mechanisms by which YYF protects against TBI-induced hematopoietic injury are unknown. More research is needed to determine whether YYF mediates ROS-related pathways in its role in radioprotection, or whether there are significant

changes to the expression of other genes and proteins that result in the protective effects of YYF.

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