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Radiation Protective Effects of *Cordyceps sinensis* in Blood Cells

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Article info

Abstract

Objective: This study investigated the radiation protective effects of *Cordyceps sinensis* (CS) extract in C57BL/6 mice.
Materials and Methods: CS powder was extracted with methanol, concentrated and re-dissolved in de-ionized water as an extract solution. The potential of the extract to eliminate hydroxyl free radicals was measured with an ultra-weak chemiluminescence analyzer. Twelve C57BL/6 mice were used for *in vivo* and *in vitro* experiments. After exposure to 0 Gy or 5 Gy whole-body γ-ray irradiation, mice in the experimental groups (3 mice/group) were fed 1 mL of a 1000 μg/mL extract daily for 3 consecutive days while mice in the control groups (3 mice/group) were given saline. After irradiation, monocytes in the peripheral blood of the mice were separated and examined for micronuclei. Tail blood was also used for leukocyte and erythrocyte counts. Bone marrow stem cells from the mice were co-cultured with a 2 μg/mL extract for the *in vitro* experiment. The expression of the *Cbfa* (C-Module DNA-Binding Factor) gene in the experimental group was then compared with the controls after 3 days.
Results: The inhibition concentration 50% (IC50) value of CS was found to be 16.6 μg/mL, indicating that it had good potential to eliminate hydroxyl free radicals. The *in vivo* experiment showed that CS was able to reduce the production of micronuclei induced by radiation. The *in vitro* experiment indicated that *Cbfa* gene expression was enhanced by CS.
Conclusion: CS was able to reduce depletion in blood cells after irradiation. The radiation protective mechanisms of CS included elimination of hydroxyl free radicals generated at the initial stage of radiolysis and an increase in *Cbfa* expression which stimulates hematopoietic stem cell differentiation. [*Tzu Chi Med J* 2007;19(4):226–232]

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1.   Introduction

Human bone marrow consists of two types of stem cells, hematopoietic stem cells and stromal cells. Hematopoietic stem cells are capable of differentiating into blood cells such as leukocytes, erythrocytes and platelets that are found in the circulation to maintain the normal functions of the blood. On the other hand, stromal stem cells (also called mesenchymal stem cells) are adhesive and are able to differentiate into osteoblasts, chondrocytes, myocytes and other types of cells. Bone marrow provides a microenvironment [1–4] composed of osteoblasts, osteoclasts and lipocytes for the growth and differentiation of hematopoietic cells. In this microenvironment, a transcription factor, Cbfa (C-Module DNA-Binding Factor), was found to be a major regulator of osteoblast differentiation [5] and hematopoietic development [6].

The typical clinical treatment for individuals with malignancies is surgery, chemotherapy, radiotherapy or a combination of these procedures. Since bone marrow is sensitive to ionizing radiation, radiotherapy can cause the death of a large number of stem cells and induce bone marrow failure [7–9]. Radiotherapy also induces side effects such as a decrease in peripheral blood cells, reduction of hematopoiesis, suppression of the body's immune response, focal scalp hair loss due to injury to follicular stem cells, and gastrointestinal syndrome as a result of radiation-induced damage to the intestinal villi [10–12].

Cordyceps is a genus of ascomycete fungi, of which the most famous species is *Cordyceps sinensis* (CS), which grows in the moth caterpillar. Spores enter the host, geminate, and ultimately kill the larva [13,14]. CS has long been used as a tonic food and herb in Chinese traditional medicine. Much research on its biological effects and related mechanisms has been reported, indicating that CS is capable of enhancing the metabolism of biomolecules (e.g. glucose and lipid), suppressing free radicals [15,16], reducing apoptosis of normal cells [15,16] while enhancing that of cancer cells [17–19], and increasing the body's immunity by promoting the activity of T cells [20], Kupffer cells [21] and macrophages [22]. However, the biological effects of CS against radiation have not been well studied [23]. Since highly reactive hydroxyl free radicals (OH) are generated immediately after radiotherapy and induce radiobiological effects, the antioxidation efficacy of CS was investigated in this study. Cell count investigation of micronuclei and reverse transcriptase–polymerase chain reaction (RT-PCR) were also performed to investigate possible radiation protective mechanisms.

2.   Materials and methods

2.1.   *Extraction of CS*

Natural CS was dried and ground to a powder, which was then extracted with methanol (Merck KGaA, Darmstadt, Germany) and further concentrated with a rotary evaporator (Heidolph Laborota Model 4000-efficient; Heidolph Elektro GmbH & Co., Schwabach, Germany). The extract was re-dissolved with de-ionized water for chemiluminescence analysis, or with saline (1:4 w/w) for animal experiments.

2.2.   *Treatment of animals*

For the *in vitro* experiment, 12 C57BL/6 mice aged 8–12 weeks were utilized. The experimental groups (3 mice/group) were exposed to 0 Gy or 5 Gy whole-body γ-ray irradiation. After irradiation, each mouse was fed 1 mL of a 1000 µg/mL CS extract daily for 3 consecutive days, while the control groups (3 mice/group) were given sterilized saline. All animal tests were performed using regulatory testing strategies for laboratory animal use and care according to the EEC directive of 1986 (86/609/EEC).

2.3.   *Peripheral cell counts*

Tail blood (15 µL) from the mice was sampled with a capillary tube containing EDTA on days 0, 1, 5, 7 and 14 after irradiation, and was then analyzed with a hemacytometer to determine the number of leukocytes and erythrocytes.

2.4.   *Hydroxyl radical scavenging potency*

Since water is abundantly present in organisms, hydroxyl free radicals are easily produced during radiolysis due to radiation exposure. Unlike superoxides, which can be eliminated by superoxide dismutase, hydroxyl radicals can react with and even damage any oxidizable molecules in their vicinity. Therefore, the potency of CS on scavenging hydroxyl radicals was evaluated using the Fenton reaction [24].

Reagents prepared included 3 µM indoxyl-β-glucuronide (IBG) dissolved with phosphate-buffered saline (PBS), 1.0 M $FeSO_4$, 3% H_2O_2 and 10 mM EDTA [25]. The above reagents were mixed by sequential addition of 0.05 mL EDTA, 1.0 mL IBG, 1.6 mL H_2O_2 and 0.1 mL $FeSO_4$ in a cylindrical quartz cup and immediately placed in the sample holder of the Ultraweak Chemiluminescence Analyzer (BJL-1-IC; Jye Horn Co., Taipei, Taiwan) calibrated with a 14C radiation source

after 30 minutes of warm-up. As the Fenton reaction was initiated, hydroxyl radicals were generated and the chemiluminescence intensity (represented by "count") increased until it reached a constant value (A_0). An aliquot of $10\,\mu L$ $1000\,\mu g/mL$ CS was added sequentially into the reagent solution each time a constant value (A_n) was reached. The inhibited percentage of radicals (inhibition%) was estimated by Equation 1 each time CS extract was added. By calculating the inhibition% versus the extract volume, the inhibitory concentration 50% (IC50; also called the median inhibitory concentration) was obtained.

$$\text{Inhibition\%} = \frac{A_0 - A_n}{A_0} \times 100\% \qquad (1)$$

2.5.   Separation of monocytes

Peripheral blood was sampled and collected from the mice by heart puncture. After 10 minutes of centrifugation at 1300 rpm, the buffy coat was collected in a 15 mL centrifuge tube, and 5 mL Ficoll 400 (Sigma-Aldrich Co., St Louis, MO, USA) was added. The monocytes were separated by further centrifugation at 2000 rpm for 15 minutes.

2.6.   Analysis of micronuclei

Monocytes separated from the peripheral blood were incubated in 12-well culture plates (1×10^4/well) with Gibco RPMI medium (Invitrogen Co., Carlsbad, CA, USA). After irradiating the monocytes with 5 Gy γ-rays, an aliquot of $50\,\mu g/mL$ phytohemagglutinin (PHA) was added to stimulate mitosis of the monocytes. However, the generated micronuclei were easily scavenged by enzymatic reactions in the cells. Therefore, cytochalasin B (Sigma-Aldrich Co.) was added into the medium ($1\,\mu g/mL$) 44 hours after culture to stop cell division during mitosis, which conserved the micronuclei and simplified observation of binucleated cells. After 24 hours, the cells were collected by centrifugation at 1300 rpm for 3 minutes and placed on a glass slide. After smearing the sample with another slide at a 45° angle, the cells were stained with 15% Giemsa's stain (Delasco Co., Council Bluffs, IA, USA) at room temperature for 10 minutes. The ratio of micronuclei ($< 1/3$ nucleus size) per 1000 binucleated cells was then estimated using an inverse microscope (Nikon TE-2000U; Nikon Corp. Instruments Co., Kanagawa, Japan).

2.7.   Bone marrow culture

The femur and tibia obtained from the mice were placed in 70% ethanol for 5 minutes and then both ends of the bone were removed with scissors. Stem cells inside the bones were flushed out with HBSS (Gibco Industries Inc., Langley, OK, USA) with a syringe and cultured in 12-well plates at a cell density of 1×10^4/well. The experimental groups were irradiated with 0 Gy or 5 Gy γ-irradiation, and then the stem cells were treated with $2\,\mu g$ /mL CS extract. The controls were divided into two groups, one group treated with the extract and the other without it.

2.8.   RT-PCR for Cbfa gene expression

On the third day of bone marrow culture, the suspended cells were transferred into a centrifuge tube and centrifuged at 1500 rpm for 5 minutes. After the supernatant was discarded, the precipitate was flushed with PBS and another centrifugation (1500 rpm for 5 minutes) was performed to collect the cells. The RNA of the cells was then extracted with RNeasy® Mini Kit (Qiagen Co., Hong Kong). RT-PCR were conducted using AMV (avian myeloblastosis virus) reverse transcriptase (Qiagen Co.), Tag DNA polymerase (Qiagen Co.), and a primer with the sequence as below for reverse transcription.

The primers (MWG-Biotech, Ebersberg, Germany) used to generate PCR probes were as follows: GAPDH, upper primer 5′CCA GCC CCA GCG TCA AGG GG 3′ and lower primer 5′GCG GGG CTC TCC AGA AAC ATC A 3′; Cbfa, upper primer 5′ACC AAG TAG CCA GGT TCA A 3′ and lower primer 5′TCT CAG TGA GGG ATG AAA TG 3′.

The transcribed cDNA was amplified with a thermocycler (Model PTC-100; MJ Research Inc., St Bruno, Quebec, Canada) using the following program: 1 cycle at 94°C for 3 minutes; 94°C for 30 seconds; 55°C for 30 seconds; 30 cycles at 72°C for 40 seconds; and 1 cycle at 72°C for 5 minutes. The final temperature was fixed at 25°C. As the reaction was finished, $2\,\mu L$ ethidium bromide was added into the product for electrophoresis in 2% agarose. The density of the band pattern after electrophoresis was compared with the control to evaluate Cbfa gene expression.

3.   Results

3.1.   Evaluation of the efficacy of antihydroxyl radicals

The intensity (expressed as the count) of the chemiluminescence generated by the Fenton reaction was inhibited by CS (Fig. 1), and this was further transformed into an inhibition% versus volume (μL) diagram (Fig. 2) with Equation 1. Using the concentration of CS, the IC50 was estimated to be $16.6\,\mu g/mL$, which
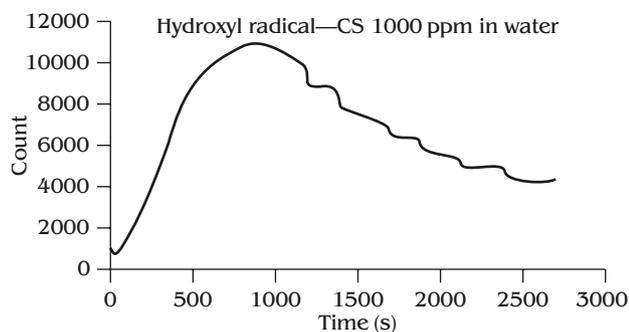
Fig. 1 — **Intensity variation of the chemiluminescence initiated by the Fenton reaction with time. The intensity increased to a constant value after the hydroxyl radicals were generated. When an aliquot of 10 μL of a 1000 μg/ mL *Cordyceps sinensis* extract was added, the intensity decreased and reached another constant level.**
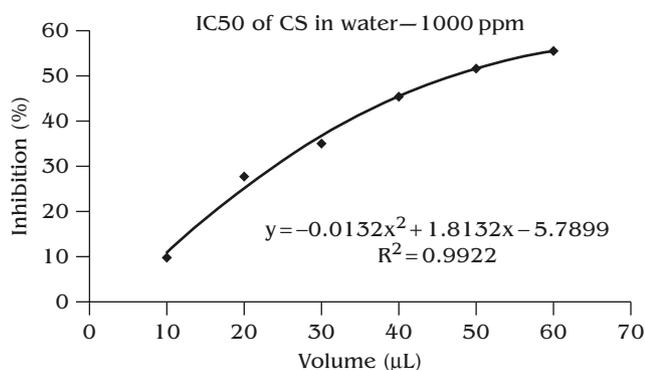

Fig. 2 — **Relationship between the inhibition percentage of hydroxyl free radicals and the volume of *Cordyceps sinensis* (CS) extract (1000 μg/mL). The CS volume (as well as CS mass) at 50% inhibition can also be obtained based on the regression curve by Bolzano's theorem. By considering the final volume of the solution (total added CS volume + original volume of the hydroxyl-radical generating solution [2.75 mL]), an accurate IC50 (16.6 μg/mL) of the CS extract could be acquired.**

Fig. 3 — **Tail blood of mice given 1 mL of *Cordyceps sinensis* extract per day for 3 consecutive days after 5 Gy γ-irradiation was sampled on days 0, 1, 5, 7 and 14, and the number of erythrocytes was analyzed with a hemacytometer. The number of erythrocytes in the control group decreased after the fifth day, while that of the experimental group remained constant.**

Fig. 4 — **The leukocyte count from tail blood of mice in the experimental and control groups after irradiation with 5 Gy γ-rays. Leukocyte counts in both groups declined rapidly after irradiation; the control count remained low after the fifth day, while that of the experimental group (dosed with *Cordyceps sinensis* extract 1 mL/day) rose.**

indicates that CS extract exhibited good efficacy in scavenging hydroxyl free radicals.

3.2. *Efficacy of CS in the protection of peripheral blood cells against radiation*

The erythrocyte count in mice after 5 Gy γ-irradiation is presented in Fig. 3. The number of erythrocytes in the peripheral blood of the control group exhibited no apparent difference in the first 5 days but declined after the fifth day and increased slightly after the seventh day. In the experimental group, the number of erythrocytes remained steady for 14 days after radiation exposure, indicating that CS can reduce decreases in erythrocytes after irradiation.
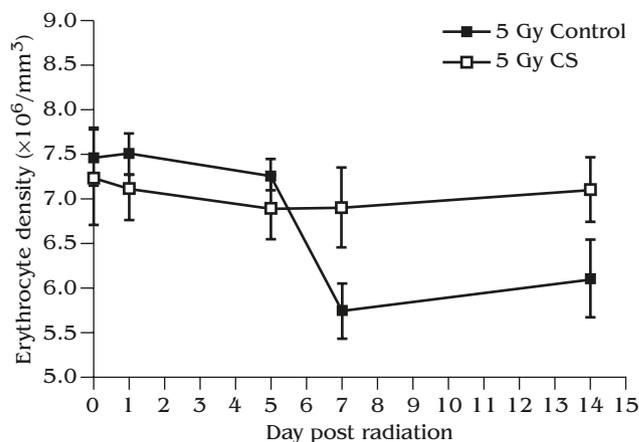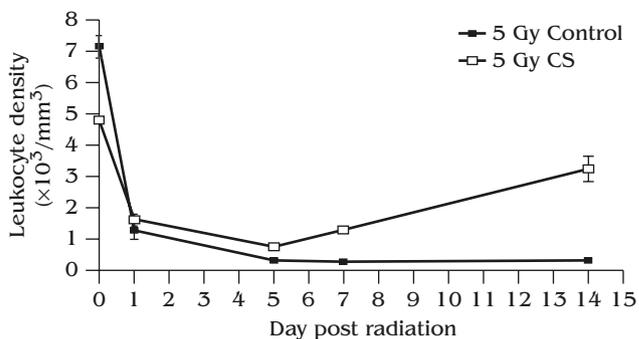
The leukocyte count in the peripheral blood of mice after 5 Gy of γ-irradiation is illustrated in Fig. 4. The number of leukocytes in both the control and experimental groups decreased abruptly after the first day of irradiation. The decrease in the controls remained until the 14th day. However, the number of leukocytes in the experimental group increased after the fifth day and continued to rise up to day 14.

3.3. *Effect of CS on micronuclei induced by radiation*

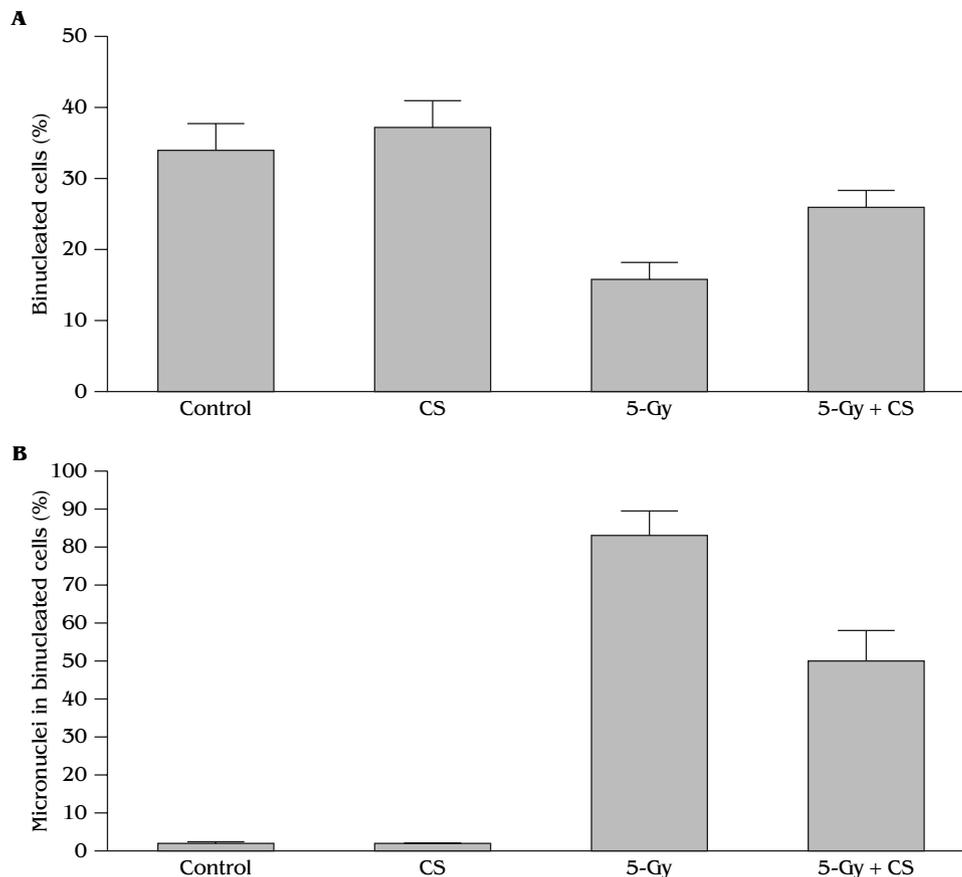Fig. 5A illustrates the percentage of binucleated cells among peripheral monocytes irradiated with 5 Gy

Fig. 5 — Effect of *Cordyceps sinensis* (CS) on the number of micronuclei induced by γ-radiation. The four groups were: Control = no irradiation or CS treatment; CS = not irradiated and treated with CS; 5-Gy = irradiated and not treated with CS; 5-Gy+CS = irradiated and treated with CS. Phytohemagglutinin (50 g/mL) was added to all separated lymphocytes. After 44 hours of culture, cytochalasin B (1 g/mL) was added into the medium. The cells were further collected after the next 24 hours of culture, and then stained with 15% Giemsa reagent for 10 minutes. (A) Percentage of binucleated cells per 1000 cells. (B) Percentage of micronuclei in the binucleated cells. The error bars indicate standard error.

γ-rays. The percentage for the non-irradiated group dosed with CS (the CS group) was 37%, showing no apparent difference from the control group that was not dosed with CS. On the other hand, the percentage in the irradiated control without CS treatment was 16% (the 5-Gy group), while that of the experimental group (irradiated and treated with CS; the 5-Gy+CS group) was 26%.

The percentage of micronuclei among the binucleated monocytes is shown in Fig. 5B. The 5-Gy group had a higher percentage (~90%) than that of the control and CS groups, as well as the 5-Gy+CS group (~50%). In the non-irradiated groups, no difference was observed between the control and CS groups.

3.4. Effect of CS on Cbfa gene expression in stromal cells

The *Cbfa* gene expression in stromal cells is illustrated in Fig. 6. The *Cbfa* gene expression in the CS

group was enhanced while that of the 5-Gy group was inhibited. According to the expression in the 5-Gy+CS group, the inhibition of *Cbfa* expression was moderated by dosing with CS extract.

4.   Discussion

Decreases in erythrocytes and leukocytes induced by γ-radiation may be reduced by dosing mice with CS extract before irradiation according to ultra-weak chemiluminescence analysis. The possible mechanisms for the radiation protection are as follows: first, CS is effective in scavenging the oxidative species, hydroxyl free radicals, as shown by an IC50 value of 16.6 μg/mL (Figs. 1 and 2). After the mice were exposed to ionizing radiation, hydroxyl free radicals were generated via breakage of the chemical bonds between hydrogen and oxygen molecules from water inside the body of the mice. With the abundance of water molecules in the body, blood cells can easily
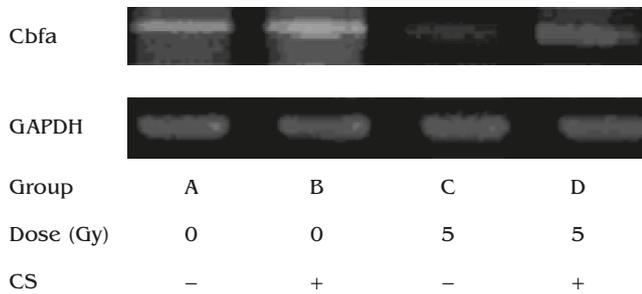
Group	A	B	C	D
Dose (Gy)	0	0	5	5
CS	–	+	–	+

Fig. 6 — Effect of *Cordyceps sinensis* (CS) on *Cbfa* gene expression in irradiated stromal cells. Total RNA in bone marrow stem cells (1×10^4/well) cultured in MEM medium was extracted on the third day after irradiation and investigated by RT-PCR and electrophoresis. The brighter the band (relative to the indicator, the GAPDH band), the higher the *Cbfa* expression. The results indicate that CS was able to enhance *Cbfa* expression. Furthermore, radiation may suppress *Cbfa* gene expression, while the suppression can be reduced by CS.

be harmed by free radicals [26,27]. Therefore, when an organism is dosed with CS before irradiation, CS may exert its protective effect against chemical effects in the initial stage and in subsequent biological effects (e.g. blood cell lesions) [28] through scavenging hydroxyl free radicals for radiation-induced oxidation reactions.

Second, CS extract is capable of stimulating cells to secrete cytokines, e.g. IL-1 [29,30], which can protect blood cells against radiation damage and therefore, can increase the survival rate of irradiated blood cells.

Third, the bone marrow microenvironment for the growth of blood cells can be stimulated by CS extract, which can enhance the differentiation of hematopoietic stem cells (Fig. 5). Although the microenvironment of bone marrow includes osteoblasts, stromal cells, reticuloendothelial cells, and lipocytes, the first two have a dominant effect on the microenvironment [31,32]. Stromal stem cells are able to secrete Cbfa (Fig. 6), which also induces differentiation of stromal stem cells. Further, hematopoietic stem cells can be stimulated to differentiate as demonstrated by the maintenance of the erythrocyte count (Fig. 3) and recovery of the leukocyte count (Fig. 4).

Micronuclei are produced in the telophase of mitosis due to breakage of the chromosome when moving to the centromere through spindles. Therefore, the percentage of peripheral monocytes containing micronuclei can be used as an indicator of chromosomal aberrations, as a higher percentage of cells with micronuclei indicates more damaged chromosomes [33]. However, the formed micronuclei are short-lived and therefore cytochalasin B was utilized to conserve the micronuclei as well as the binucleated cells generated. According to Fig. 5, the percentages of cells with micronuclei among binucleated cells in the control group (~2.0%) and the CS group (~1.2%) were lower than those in the 5-Gy (~87%) and 5-Gy+CS groups (~52%). Among the irradiated groups, the CS-treated group had a considerably lower percentage of micronuclei. This indicates that CS extract has a noticeable effect in reducing chromosome damage after irradiation, which may be ascribed to its efficacy in stimulating cytokines protecting cells against lesions [29,30] due to irradiation.

In conclusion, chemiluminescence analysis of CS extract illustrated that the IC50 of hydroxyl radicals was 16.6 μg/mL. This suggests that CS is effective in scavenging hydroxyl free radicals and can protect blood cells from initial oxidation reactions after γ-ray irradiation. After CS was administered, the irradiated mice maintained a steady erythrocyte level. The leukocyte levels were able to recover earlier and had increased within 14 days. Cell differentiation of the bone marrow microenvironment was enhanced by CS as shown by *Cbfa* expression in the stromal cells from the mice dosed with CS. Chromosome damage due to irradiation was also reduced in the CS group, as indicated by the reduced percentage of binucleated cells among the peripheral monocytes. The results indicated that CS was efficacious in protecting blood cells from radiation damage and in reducing side effects during radiotherapy.

Acknowledgments

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