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Black Hoof Medicinal Mushroom *Phellinus linteus* (Agaricomycetes) Extracts Protect Against Radiation-Induced Hematopoietic Abnormality in Mice

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ABSTRACT: We investigated the effects of *Phellinus linteus* extracts (PLEs) against radiation damage in mice. First, BALB/c mice were irradiated once with γ-rays at 4, 5, 6, or 8 Gy and allowed to recover for 20 days. Results reveal that 8-Gy radiation caused death in 100% of mice on day 13, and 6-Gy radiation caused death in 86.7% of mice (13/15) at the end of the experiment, whereas 4- and 5-Gy radiation did not result in any death. We then used 5-Gy γ-ray radiation to examine the protective effects of PLEs. Mice were orally administered a PLE (500, 1000, and 1500 mg/kg) daily for 2 weeks before radiation and for 6 weeks after radiation. γ-Ray radiation significantly decreased body weight starting from week 2 after radiation. Supplementation with a median and high dose of PLE significantly restored body weights starting at weeks 5 and 3, respectively. The radiation-protective agent WR2721 (200 mg/kg intraperitoneally) restored body weights starting at week 4. White blood cells, platelets, red blood cells, and hemoglobin were significantly decreased by radiation, and PLEs (primarily at high doses) and WR2721 significantly prevented hematologic abnormality. These results suggest that PLE has potential as a radioprotective agent.

KEY WORDS: γ-ray radiation, hematologic parameters, medicinal mushrooms, *Phellinus linteus* extracts

ABBREVIATIONS: PLE, *Phellinus linteus* extract; RBC, red blood cell; WBC, white blood cell

I. INTRODUCTION

Radiation is ubiquitous in daily life and can be divided into ionizing and nonionizing based on whether it ionizes atoms. Ionizing radiation includes ultraviolet, X-ray, γ-ray, α-ray, and β-ray radiation, whereas nonionizing radiation includes visible light, microwaves, radio waves, and infrared light. Studies have indicated that exposure to high doses of radiation cause various biological damage in mammals, such as hematopoietic dysfunction, immunosuppression, infection, hemorrhage, and even death.1–3 It is critical to search natural resources for possible radioprotectants that protect biological systems against radiation-induced damage.4

The black hoof mushroom, *Phellinus linteus* (Berk. et M.A. Curt.) Teng (Hymenochaetaceae, Agaricomycetes), is one of the traditional medicinal mushrooms that has been used for centuries in Asian countries such as Japan, Korea, China, and Taiwan. Several bioactive components such as polysaccharides, proteoglycans, hispidin, and hispolon have been identified from *Ph. linteus* and have several biological functions, including immunomodulation, anticancer, antioxidant, and hepatoprotective effects.5 However, little is known about whether *Ph. linteus* has protective effects against radiation-induced injury.

In this study, we aimed to investigate the effects of γ-ray radiation on survival rates in BALB/c mice. The mice were irradiated once with γ-rays at 4, 5,
6, and 8 Gy and allowed to recover for 20 days to determine the survival rates. We then selected a radiation dose that did not cause mouse death to examine the protective effects of Ph. linteus extracts (PLEs) on radiation-induced abnormal hematologic parameters. The mice were orally administered PLE (500, 1000, and 1500 mg/kg) daily for 2 weeks before radiation, after which oral administration of PLE was continued for an additional 6 weeks. WR2721, a known radioprotectant, was used as the positive control and administered via intraperitoneal injection 30 minutes before radiation.

II. MATERIALS AND METHODS

A. PLE Preparation

The freeze-dried powder of Ph. linteus mycelia was provided by Grape King Inc. (Zhongli City, Taoyuan County, Taiwan). Ph. linteus mycelia with the voucher specimen BCRC PL003 was deposited at the Biotechnology Center of Grape King Inc. To prepare the PLE, the mycelia 4 (100 g) was refluxed with 1900 mL of 95% ethanol or hot water, with constant stirring at 110 rpm, 3 times (2 hours each time). After centrifugation, the supernatants were collected, concentrated, and dried under a vacuum. The recovery ratios for hot water and ethanol extracts were 28.1% and 12.9%, respectively. PLE was obtained by mixing the hot water extract with the ethanol extract at a ratio of 1:1 (w/w).

B. Analysis of Total Flavonoids, Polyphenols, and Polysaccharides in PLE

The total flavonoid and polyphenol contents in PLE were determined using the aluminium chloride method and the Folin-Ciocalteu method, respectively. The calibration curves were established using as reference compounds catechin for flavonoids and gallic acid for polyphenols. The polysaccharide content in PLE was determined using the phenol-H$_2$SO$_4$ method. A calibration curve was established using glucose as the reference compound.

C. Animals and Treatment

Six-week-old male BALB/c mice with an average body weight of 20 g were purchased from BioLASCO Taiwan Co., Ltd (Yilan, Taiwan). This study’s protocol was approved by the Animal Research Committee of National Chung Hsing University (IACUC approval no. 99-47). The mice were housed in cages with a controlled temperature (25 ± 2°C) and humidity (65 ± 5%) with 12-hour light/12-hour dark cycles, and they were accommodated for 1 week after arrival. To select the appropriate radiation dose, the mice were randomly divided into 5 groups (n = 15 in each group): group I, control; group II, 4-Gy radiation; group III, 5-Gy radiation; group IV, 6-Gy radiation; and group V, 8-Gy radiation. The mice were irradiated once with $\gamma$-rays and allowed to recover for 20 days to determine the survival rates.

We then selected a median radiation dose of 5-Gy irradiation to study the protective effect of PLE on radiation. The mice were randomly divided into 6 groups (n = 15 in each group) based on body weight: group I, control (no radiation); group II, radiation; group III, radiation plus low-dose PLE (500 mg/kg); group IV, radiation plus median-dose PLE (1000 mg/kg); group V, radiation plus high-dose PLE (1500 mg/kg); group VI, radiation plus WR2721 (200 mg/kg). PLE (500, 1000, and 1500 mg/kg) was orally administered to the mice daily for 2 weeks before irradiation once with 5-Gy $\gamma$-rays, after which oral administration with PLE was continued for an additional 6 weeks, 3 times per week. WR2721 was used as the positive control and was administered by intraperitoneal injection 30 minutes before irradiation. During the accommodation and experimental periods, the mice were supplied ad libitum water and a standard rodent diet (Lab 5001; Purina Mills) containing 59.8% carbohydrate, 23.4% protein, and 4.5% crude fat, as indicated by the supplier. The body weights of mice were measured once per week.

D. Complete Blood Count

At 48 hours, 14 days, and 42 days after irradiation, blood samples were collected from the
retro-orbital plexus into an Eppendorff tube containing K$_3$EDTA and centrifuged (400 × g for 10 minutes) to obtain plasma. The white blood cells (WBCs), red blood cells (RBCs), hemoglobin, and platelets in plasma were analyzed using KX-21N Automated Hematology Analyzer (CLIAwaived Inc., San Diego, CA).

E. Gamma Radiation

A γ-radiation source from an Elekta Synergy system with the linear accelerator (provided by Jen-Ai Hospital Taichung Branch, Taichung, Taiwan) was used for the radiation exposure experiment. The mice were placed in well-ventilated boxes (15 mice/box) and subjected to whole-body γ-radiation at different doses (4, 5, 6, and 8 Gy) at room temperature. The distance from the radiation source to the skin was 7 cm.

F. Statistical Analysis

Values are expressed as mean ± SD and were analyzed using 1-way analysis of variance followed by the Fisher protected least significant difference test for comparisons of group means when the F ratios were significant ($P < 0.05$). All statistical analyses were performed using SPSS for Windows, version 10 (SPSS, Inc.); a $P$ value < 0.05 is considered statistically significant.

III. RESULTS AND DISCUSSION

A. Effects of Different Doses of γ-Ray Radiation on Survival Rates in Mice

In mice radiated once with γ-rays at 4, 5, 6, or 8 Gy and allowed to recover for 20 days, we found that irradiation with 8-Gy γ-rays resulted in death in 26.7% (4/15 mice) on the 9th day and in 100% on the 13th day after irradiation (Fig. 1). Irradiation with 6-Gy γ-ray caused death in 13.3% (2/15 mice; $P < 0.05$) on the 13th day and in 86.7% (13/15 mice, $P < 0.05$) at the end of experiment (20th day after irradiation) (Fig. 1). In addition, irradiation of mice with 6- and 8-Gy γ-ray caused radiation sickness within 5–7 days after irradiation; the symptoms included weight loss, poor appetite, blurred vision, and alopecia. The whole-body irradiation of male Swiss albino mice with 10 Gy of Co$^{60}$ γ-ray radiation resulted in a death rate of 89% within 10 days as a result of the functional failure of the gastrointestinal tract; the remaining 11% of mice died within the next 7 days with abnormal hematopoietic syndromes. Bone marrow stem cells and peripheral blood cells are more sensitive to radiation damage and have a longer transit time than intestinal cells; therefore the gastrointestinal syndrome seems to occur earlier than the bone marrow syndrome.

By contrast, radiation with γ-rays at 4 and 5 Gy did not cause the death of any mice during the 20-day recovery period (Fig. 1), and the radiation sicknesses outlined above were weaker than in mice exposed to 6- and 8-Gy irradiation. Thus, we chose a median dose between 4 and 6 Gy (i.e., 5 Gy) to determine the protective effects of PLE against radiation-induced abnormal hematologic parameters.

B. Effects of PLE Supplementation on Radiation-Induced Body Weight Loss in Mice

Body weight loss is one of the effects caused by radiation. To examine the protective effects of
PLE, we orally administered PLE to the mice at 500, 1000, and 1500 mg/kg daily for 2 weeks before radiation once (5-Gy γ-ray), after which oral administration of PLE 3 times/week was continued for an additional 6 weeks. WR2721 was used as the positive control by intraperitoneal injection 30 minutes before radiation. The body weights of mice were measured weekly. R, radiation alone; R + H, radiation + high-dose PLE; R + L, radiation + low-dose PLE; R + M: radiation + median-dose PLE. Values (mean ± SD) in the same column not sharing an alphabetic letter are significantly different (P < 0.05).

C. Effects of γ-Ray Radiation and WR2721 Treatment on Hematologic Parameters in Mice

The hematopoietic system, especially the bone marrow, is most sensitive to ionizing radiation; the major symptoms of hematopoietic damage caused by radiation, even at low doses, include adipose tissue hyperplasia, cell apoptosis and depletion, reduced peripheral blood cells, and hematopoietic progenitor cells. Here we found that 5-Gy γ-ray irradiation of mice significantly decreased the numbers of WBCs, RBCs, hemoglobin, and platelets 48 hours following irradiation compared with the control mice (Table 2). These levels continued to decrease at 14 days after irradiation, except for the levels of WBCs, which were lowest at 48 hours following irradiation. All these levels showed some recovery but were still significantly lower than those of control mice (Table 2).

Several radioprotective agents have been identified to decrease radiation-induced free radical levels within cells. WR2721, also called amifostine, is the only US Food and Drug Administration–approved radioprotector and has been used to reduce the incidence of xerostomia in patients with head and neck cancer receiving radiotherapy. The current results reveal that WR2721 administration significantly restored the body weights of mice starting at 4 week after radiation compared with those of mice irradiated with γ-rays alone (Table 1). In addition, treatment with WR2721 in general significantly increased the levels of WBCs, RBCs, hemoglobin, and platelets 14 days after irradiation compared with those of mice irradiated with γ-rays alone, and it completely restored these levels to the control levels at 42 days after irradiation (Table 2). The results of WR2721 treatment confirm the effectiveness of WR2721 as a protective agent against radiation injury.
TABLE 2: Effects of *Phellinus linteus* Extracts on Radiation-Induced Abnormal Hematologic Parameters in Mice*

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBC (10^3/μL)</th>
<th>RBC (10^3/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 Hours</td>
<td>14 Days</td>
</tr>
<tr>
<td>Control</td>
<td>4.5 ± 0.5^a</td>
<td>4.7 ± 0.6^a</td>
</tr>
<tr>
<td>R</td>
<td>0.2 ± 0.1^b</td>
<td>0.6 ± 0.1^b</td>
</tr>
<tr>
<td>R + L</td>
<td>0.6 ± 0.1^c</td>
<td>0.7 ± 0.1^b</td>
</tr>
<tr>
<td>R + M</td>
<td>0.6 ± 0.1^c</td>
<td>0.6 ± 0.1^b</td>
</tr>
<tr>
<td>R + H</td>
<td>0.7 ± 0.1^c</td>
<td>0.7 ± 0.1^b</td>
</tr>
<tr>
<td>R + WR2721</td>
<td>0.7 ± 0.1^c</td>
<td>1.1 ± 0.2^c</td>
</tr>
</tbody>
</table>

Hemoglobin (g/dL) | Platelets (10^9/μL)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.4 ± 1.7^a</td>
</tr>
<tr>
<td>R</td>
<td>7.5 ± 1.0^b</td>
</tr>
<tr>
<td>R + L</td>
<td>8.0 ± 0.4^c</td>
</tr>
<tr>
<td>R + M</td>
<td>8.6 ± 0.4^c</td>
</tr>
<tr>
<td>R + H</td>
<td>8.1 ± 0.9^b</td>
</tr>
<tr>
<td>R + WR2721</td>
<td>8.5 ± 0.5^b</td>
</tr>
</tbody>
</table>

*Mice were orally administered *Phellinus linteus* extracts (PLE) daily for 2 weeks before radiation once (5-Gy γ-ray), after which oral administration of PLE 3 times/week was continued for an additional 6 weeks. WR2721 was used as the positive control by intraperitoneal injection 30 minutes before radiation. Complete blood counts were measured at 48 hours, 14 days, and 42 days after radiation. R, radiation alone; R + H, radiation + high-dose PLE; R + L, radiation + low-dose PLE; R + M: radiation + median-dose PLE; RBC, red blood cell; WBC, white blood cell. Values (mean ± SD) in the same column not sharing an alphabetic letter are significantly different (P < 0.05).

D. Effects of PLE Supplementation on Radiation-Induced Abnormal Hematologic Parameters in Mice

The degree of hematopoietic dysfunction and recovery efficacy of abnormal hematopoietic symptoms has been shown to play an important role in determining the therapeutic efficiency of diseases caused by radiation. The current results reveal that pretreatment of mice with PLE significantly improved radiation-reduced levels of WBCs, RBCs, hemoglobin, and platelets. PLE significantly and dose-dependently increased radiation-reduced WBC levels at 48 hours, but not at 2 weeks, after radiation (Table 2). At the end of the experiment (42 days after irradiation), only the high-dose PLE supplementation significantly increased radiation-reduced WBC levels compared with those of mice irradiated with γ-rays alone (i.e., without PLE supplementation), though the levels were still significantly lower than those of control mice (Table 2). PLE supplementation in general did not have a significant effect on RBC and hemoglobin levels either 48 hours or 2 weeks after irradiation (Table 2). However, high-dose PLE supplementation restored RBC and hemoglobin levels to those of control mice at the end of experiment. Similarly, PLE supplementation in general did not have a significant effect on platelet levels at either 48 hours or at 2 weeks after irradiation compared with those of mice irradiated with γ-rays alone, but the high-dose PLE completely restored the platelets to the control level at the end of the experiment (Table 2).
TABLE 3: Amounts of Total Flavonoids, Polyphenols, and Polysaccharides in Phellinus linteus Extracts

<table>
<thead>
<tr>
<th>PLEs</th>
<th>Contents (mg/100 mg dried PLE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>Polyphenols</td>
<td>11.9 ± 0.8</td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>62.6 ± 5.2</td>
</tr>
</tbody>
</table>

PLE, Phellinus linteus extracts.

E. Contents of Total Flavonoids, Polyphenols, and Polysaccharides in PLE

Several bioactive compounds including flavonoids, polyphenols, and polysaccharides isolated from plants, tea, and mushrooms have been shown to exhibit protective effects against radiation-induced damage. This study shows that the amounts of total flavonoids, total polyphenols, and polysaccharides in PLE were 3.0 ± 0.4, 11.9 ± 0.8, and 62.6 ± 5.2 mg/100 mg, respectively (Table 3). These components have previously been identified in Ph. linteus and are suggested to have biological functions including immunomodulation, antitumor, antioxidant, and hepatoprotective effects. The relatively abundant polysaccharides in PLE, along with flavonoids and polyphenols, may contribute to the radioprotective effects of PLE.

IV. CONCLUSIONS

This study demonstrated that PLE effectively improves γ-ray radiation-induced abnormal hematologic parameters in mice, suggesting that PLE could work as a radioprotectant or an adjuvant for radiotherapy (Fig. 2). Because γ-ray radiation is known to cause oxidative damage to biological systems, it is possible that the protective effect of PLE against radiation-induced hematopoietic abnormality may be attributed to the antioxidant and cytoprotective activities of Ph. linteus, which contains bioactives such as flavonoids, polyphenols, and polysaccharides. Further investigations of the possible radioprotective components of PLE are warranted.

FIG. 2: Scheme of the proposed protective action of Phellinus linteus extracts (PLEs) against radiation-induced hematopoietic abnormality in mice.  A result of bone marrow injury by γ-radiation.  Protection by PLE.

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