Antioxidant and Immuno-Enhancing Effects of *Echinacea purpurea*

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We studied the protective effects of *Echinacea purpurea* against radiation by evaluating changes in the peripheral blood cell count and peripheral blood antioxidant activity. *E. purpurea* administration had a suppressive effect on radiation-induced leukopenia, especially on lymphocytes and monocytes, and resulted in a faster recovery of blood cell counts. Mouse peripheral blood antioxidant activity was increased by *E. purpurea*, and a relationship between the suppressive effect on radiation-induced leukopenia and the antioxidant effect was suggested. Furthermore, we reviewed the evidence of augmentation of found in this study humoral immunity. The effecst of immune activation by *E. purpurea* were investigated by measuring total immunoglobulin (IgG, IgM). The radioprotective effects of immune activation by *E. purpurea* were investigated by measuring T lymphocyte subsets in the peripheral blood of mice following whole-body irradiation. *E. purpurea* activates macrophages to stimulate IFN-γ production in association with the secondary activation of T lymphocytes, resulting in a decrease in IgG and IgM production. Cytokines released from macrophages in mouse peripheral blood after *E. purpurea* administration activated helper T cells to proliferate. In addition, it is reported that activated macrophages in association with the secondary T lymphocyte activation increases IFN-γ production and stimulates proliferation of cytotoxic T cells and suppressor T cells. We think that CD 4 and CD 8 subsets were more immunologically enhanced by *E. purpurea* than helper T cells and suppressor T cell these results reflect activation. In addition, we think that these results reflect cell-mediated immune responses.

Key words *Echinacea purpurea*; immunology; radiation protection; T lymphocyte; antioxidant

Echinacea purpurea has been used for many years by indigenous people of North America as a panacea for a variety of diseases including infections, trauma, inflammation, and fever.[1)] This species of echinacea is a perennial plant of the purple barren Compositae, which is indigenous to limited areas of the western and central desert areas of North America and is currently grown worldwide as an ornamental plant, and for use as a food and medicinal agent. The US Food and Drug Administration (FDA) recognizes it as a food, while Commission E (Task Force E of the Federal Bureau of Health of Germany) approved it as medicine. Radiation can have tremendous therapeutic benefits for humans; however, it is also associated with the risk of serious adverse effects. Examples of radiation-protective agents that have been used clinically include: SH compounds, such as cysteine and WR-2721 (amifostine), which remove radicals produced by radiation and thereby protect the body from the indirect effects of radiation,[2,3)] granulocyte colony-stimulating factor (G-CSF), which prevents immunosuppression from radiation exposure; and antiimmunosuppressives, such as OK-432.[4,5)] However, these agents have the potential to cause serious adverse effects, particularly when combined with other medications. It has been reported that *E. purpurea* has an interferon (IFN)-like effect, activating macrophages and inducing the production of interleukin (IL)-1 and IFN.[6)] Recently, the ability of *Echinacea* sp. to alleviate allergies and AIDS has been studied. See *et al.*[7)] reported that *Echinacea* sp. enhanced some immune functions in both healthy people and AIDS patients. There are nine known species of *E. purpurea* Therapeutic effects have been reported in three species, *E. purpurea*, *E. pallida*, and *E. angustifolia*, which are all used as medical herbs.[8)] Among their components, echinacin, a peculiar glucose chain derived from *E. purpurea*, has been reported to attach to the surface of T cells and macrophages, which protect the body from viral attack, and to activate them.[9,10)] Previous studies developed radioprotective agents to protect from the indirect effects of radiation by eliminating free radicals produced in response to radiation[2,3)] and immunostimulants to inhibit immune suppression.[4,5)] However, the toxicity and side effects of such agents have been critical problems. Certain medicinal herbs and their active constituents may be useful as radioprotective agents and be less toxic than their nonherbal counterparts.

We focused on hematocytes as markers to evaluate the protective effects of *E. purpurea* against irradiation and examined blood cells counts over time and antioxidant activity in the peripheral blood. We found that *E. purpurea* administration inhibits the reduction in white blood cells, especially lymphocytes and monocytes, after exposure to radiation. Therefore in this study we investigated the radioprotective effects of *E. purpurea* by measuring total immunoglobulin, IgG, IgM, and T lymphocyte subsets in the peripheral blood.

MATERIALS AND METHODS

Experimental Animals Five-week-old male ICR [Crj:CD-1 (Swiss Hanchka)] mice with a mean weight of 18—20 g were purchased from Japan SLC Inc. and kept under standard conditions (room temperature 22±3 °C, humidity 60%) with free access to food (CA-1, Japan Clare, Inc.) and drinking water (Top-water). The mice were acclimated to the breeding and experimental environment for 1 week prior to the experiments. The experimental animals for the studies on IgG and IgM in peripheral blood were 6-week-

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old male C3H/HeNCrj mice (mean body weight, 18—24 g) purchased from Charles River, Japan. For the studies on T lymphocytes, 3-week-old male C57BL/6crSlc mice (mean body weight, 8—13 g) purchased from Japan SLC Inc. were used.

E. purpurea The *E. purpurea* specimens used in this study were imported from the U.S.A. in a refrigerator. Whole plants of *E. purpurea* MOENCH in the flowering season (July) were pressed, and then the extracted juice was dried and refined to a powder, which was used for this study.

The *E. purpurea* dried powder was suspended in saline and the suspension was then administered into the abdominal cavity of mice at a dose of 360 mg/kg every other day. Mice were used for experiments after at least 3 weeks of *E. purpurea* administration. *E. purpurea* administration continued until the end of the experiments.

Administcation of Radiation Two Gy of whole-body X-ray irradiation was administered to each mouse using an X-ray generator designed for animal use (Phillips, Inc.). The table was rotated at a constant speed so that the mouse, restrained in a plastic jig, was irradiated evenly. The conditions for irradiation were: source voltage, 200 kV; rate of radiation, 0.35 Gy/min; and supplemental filter, 0.1 mm Cu+1 mm Al.

Measurement of Peripheral Blood Cell Counts in Mice Changes in peripheral blood cell counts were studied in 4 groups of mice: a control group, which was administered saline; an *E. purpurea* only group; an irradiation only group; and an *E. purpurea* and irradiation group, which was irradiated with 2 Gy of X-rays after administration of *E. purpurea* (*E. purpurea*+2 Gy). Ten mice from each group were used in the experiment.

The tail vein of each mouse was incised with a Spitz knife and 10 μl of peripheral blood was collected with a capillary tube. The blood cell count was performed with an automated blood cell counter (Celltac-α MEK-6318, Nippon Koden Inc.). The numbers of peripheral leukocytes, lymphocytes, granulocytes, and monocytes, which all have relatively high sensitivity to radiation, and primary cells of the immune system were counted. To observe changes in the peripheral blood cell counts, measurements were done on the day preceding irradiation and at 3 h, 12 h, 24 h, 3 d, 7 d, 15 d, and 30 d after irradiation. Statistical analysis was performed using a parametric ANOVA test among the groups to determine significant differences in blood cell counts for each group.

Measurement of Serum SOD Activity in Mouse Peripheral Blood Antioxidant activity was studied in two groups of mice: a control group that was administered saline and an *E. purpurea* group (n=10 per group).

Serum SOD activity was measured using the nitroblue tetrazolium (NBT) reduction method with a SOD Activity Detection Kit (Wako Pure Chemical Ind., Ltd.). The NBT reduction method measures SOD activity using NBT as a detector for O_2^-, coupling an O_2^- production reaction (xanthine–xanthine oxidase) with the disparity reaction by SOD, and measuring the rate of decline in reduction of coloration by O_2^- as the rate of inhibition. This method is suitable for the quantitative measurement of antioxidant activity.[11] The basic measurement protocol was as follows. In anesthetized mice, whole blood was collected from the heart using a syringe (Terumo Company) with a 23-gauge needle, heparin (5 units/ml) was added, and the serum was separated from whole blood by centrifugation at 1500 rpm for 15 min. Next, 10 μl/well of sample was added to a 96-well microplate. The samples were specimen (S), blind (Bl), specimen-blind (S-Bl), and reagent-blind (Bl-Bl). The serum for specimen (S) and specimen-blind (S-Bl) and distilled water for blind (Bl) and reagent-blind (Bl-Bl) were added. After the addition of the samples, 100 μl/well of the coloration reagent [0.1 M phosphate buffer, pH 8.0; 0.4 mM xanthine; and 0.24 mM NBT] was added and the plates were stirred for 1 min. After stirring, 100 μl/well of the enzyme solution (0.1 M phosphate buffer, pH 8.0; xanthine oxidase, 0.049 units/ml) for the specimen (S) and blind (Bl) samples, and 100 μl/well of the blank solution (0.1 M phosphate buffer, pH 8.0) for the specimen-blind (S-Bl) and reagent-blind (Bl-Bl) samples were added and stirred for 1 min, followed by incubation at 37 °C for 28 min. After incubation, 20 μl/well of the reaction-quenching reagent (69 mM sodium dodecyl sulfate) was added to each sample, stirred for 5 min, and the absorbance was measured using a microplate reader MPR A4 (Toyo Sotatsu Inc.) at a wavelength of 560 nm. SOD activity was determined from the absorbance according to the formula (1)

$$\text{SOD activity (inhibition rate \%)} = \frac{(E_{Bl}-E_{Bl\text{-}Bl})-(E_S-E_{S\text{-}Bl})}{(E_{Bl}-E_{Bl\text{-}Bl})} \times 100 \quad (1)$$

where E_S is absorbance of specimen, E_{Bl} absorbance of blind, $E_{S\text{-}Bl}$ absorbance of specimen-blind, and $E_{Bl\text{-}Bl}$ absorbance of reagent-blind.

Significant differences in SOD activity between group pairs were determined using a nonparametric Wilcoxon test.

Blood Analysis Whole blood was collected from mouse hearts by puncture with a 23-G needle under anesthesia, mixed with heparin, and either centrifuged (15 min at 1500 rpm) to separate serum for the immunoglobulin studies, or suspended 1 : 1 in PBS and then processed as described below for the T lymphocyte studies.

Measurement of Total IgG and IgM in Mouse Peripheral Blood Mice were divided into two groups: the control group, to which physiologic saline was given, and the IgG and IgM groups, to which *E. purpurea* was given. Each group comprised 10 mice.

Total serum IgG and IgM were measured using enzyme-linked immunosorbent assay (ELISA) with a mouse IgG ELISA quantification kit and a mouse IgM ELISA quantification kit (Bethyl Laboratories Inc., Montgomery, TX, U.S.A.).

In a 96-well microplate, 100 μl of the solid-phase antibody (affinity-purified goat anti-mouse IgG-Fc or IgM antibody) diluted 100-fold with coating buffer (0.05 M sodium carbonate, pH 9.6) was dispensed into each well and incubated at room temperature for 60 min. After incubation, the coating buffer was discarded and the wells were rinsed two times with washing buffer (50 mM Tris, 0.14 M NaCl, Tween 20, pH 8.0). Then, 200 μl of postcoat solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) was poured into each well to solidify the solid-phase antibody. After incubation at room temperature for 30 min, the postcoat solution was discarded and the wells were rinsed twice with washing buffer. Serum (100 μl) was diluted 50-fold with the sample diluting solution (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH

8.0) or known concentrations of standard serum (mouse IgG or IgM reference serum) were dispensed into the wells and incubated at room temperature for 60 min. After incubation, the serum was discarded and the wells were washed four times with washing buffer. In each well, 100 μl of enzyme-labeled antibody (HRP-conjugated goat anti-mouse IgG-Fc or HRP-conjugated goat anti-mouse IgM antibodies), diluted at 1 : 120000 with conjugate diluting solution (same composition as sample diluting solution), was dispensed for enzymatic reaction, and after incubation for 60 min, the enzyme solution was decanted and the wells were washed four times.

After rinsing, 100 μl of enzyme substrate solution (3,3,5,5-tetramethyl benzidine TMB) was dispensed and incubated for 15 min to develop color. After 100 μl of stop solution (2 M H_2SO_4) was dispensed into each well, optical density (OD) was measured on a MPR A4 microplate reader (Toyosohtatsu, Japan) at a reference wavelength of 450 nm. Total serum IgG and IgM concentrations were measured after a standard curve with the ODs of standard sera was drawn.

For statistical analysis, because the concentrations of total serum IgG and IgM showed normal distribution, the parametric *t*-test was used to compare the two groups.

Measurement of CD3-, CD4-, and CD8-Positive T Lymphocytes in Peripheral Blood Lymphocytes were separated using the gravity centrifugation method.[27] Lymphocyte separating solution (5 ml; sodium hypaque, Ficoll 400; specific gravity, 1.0875±0.0005 at 25 °C) was added into a 15-ml sample tube, onto which 5 ml of cell suspension was carefully loaded. After centrifugation at room temperature (15—20 °C) at 500 ***g*** for 20 min, plasma in the supernatant was collected to extract lymphocyte subsets. After addition of PBS (pH 7.2, without Ca^{2+} or Mg^{2+}) supplemented with 10% inactivated FBS (heat-inactivated at 56 °C for 30 min) and red blood cell lysing solution, the mixture was centrifuged at room temperature at 400 ***g*** for 10 min. The supernatant was collected and the cells were resuspended and washed twice in PBS containing FBS. Lymphocytes were resuspended in PBS prior to analysis.

Flow cytometry reagents for lymphocyte subset measurement were added into the lymphocyte suspension in PBS, and the mixture was stained for immunofluorescence for about 30 min at 4 °C in a dark room. After the reaction, the solution was rinsed three times with PBS, and CD3, CD4, and CD8 subsets were analyzed with a FACS Caliber flow cytometer (Becton Dickinson).

To analyze T lymphocyte subsets, Multicolor Flowcytometry (FCS) System (Santa Cruz Biotechnology Inc.) was employed and CD3-, CD4-, and CD8-positive T lymphocytes in the peripheral blood were counted in three-color flow cytometry using anti-CD3-PE-Cy5.5, anti-CD4-FITC, and anti-CD8-PE.

RESULTS

Changes in Mouse Peripheral Blood Cell Counts The study of cytopenia following irradiation (Figs. 1A—D) showed that the number of leukocytes in the irradiation only group (2 Gy group) declined markedly, while the decline was suppressed in the *E. purpurea* and irradiation group, although the suppression was not statistically significant ($p=0.06$). *E. purpurea* administration also suppressed irradiation-induced cytopenia of leukocytes. However, there was a slight difference in the influence due to differences in radiosensitivity and the lifespan of the cells. In contrast to the effects observed for other blood cells, granulocyte counts were transiently increased by irradiation, due to an influx from the reserve blood pool in response to cytopenia. The degree of this increase was proportional to the degree of cytopenia.

The study of blood cell count recovery after irradiation showed that leukocyte count recovery after irradiation occurred significantly faster in the *E. purpurea* and irradiation group than in the irradiation only group ($p<0.01$). Although there is little difference in radiosensitivity and lifespan of among lymphocytes, granulocytes, and monocytes, the recovery in the number of these cell types after irradiation tended to significantly faster with *E. purpurea* administration ($p<0.05$).

SOD Activity in Mouse Peripheral Blood The measurement of mouse serum SOD activity using the NBT reduction method (Table 1) revealed greater SOD activity in the *E. purpurea* group than in the control group, and a significant increase in SOD activity with *E. purpurea* administration was noted ($p<0.05$).

Total IgG and IgM in Mouse Peripheral Blood A standard curve was generated based on the OD of mouse IgG standard solutions of known concentrations, and total IgG in mouse serum was measured (Table 2). Compared with the control group, total serum IgG was significantly reduced in the *E. purpurea* group ($p<0.05$), indicating that *E. purpurea* suppressed IgG production.

Similarly, a standard curve based on the ODs of mouse IgM standard sera of known concentrations was used to quantify IgM in mouse serum (Table 1). ELISA revealed that the amount of IgM in mouse serum was significantly less in the *E. purpurea* group compared with the control group ($p<0.05$), indicating that *E. purpurea* suppressed IgM production.

Analysis of T Lymphocyte Subsets in Mouse Peripheral Blood On the cytogram, the lymphocyte fraction was gated, so that CD4-positive ($CD3^+CD4^+$) and CD8-positive ($CD3^+CD8^+$) cells were counted. First, CD4-positive cells were counted with flow cytometry for comparison. As shown in Fig. 2A, the number of CD4-positive cells was increased (by 93%) in only the *E. purpurea* group. Similarly, the number of CD4-positive cells increased by 40% in the *E. purpurea* with irradiation group compared with the irradiation alone group. This indicates that *E. purpurea* administration increased CD4-postive cells, *i.e.*, helper T cells, in the peripheral blood.

CD8-positive cells were also counted with flow cytometry. As shown in Fig. 2B, CD8-positive cells were increased by 201% in the *E. purpurea* group compared with the control group. Compared with the irradiation alone group, CD8-positive cells were decreased by 33% in the *E. purpurea* with irradiation group. This indicates that *E. purpurea* administration increases CD8-positve cells, *i.e.*, suppressor T cells and killer T cells, in the peripheral blood. However, in the *E. purpurea* with irradiation group, irradiation decreased the number of CD8-positive cells.

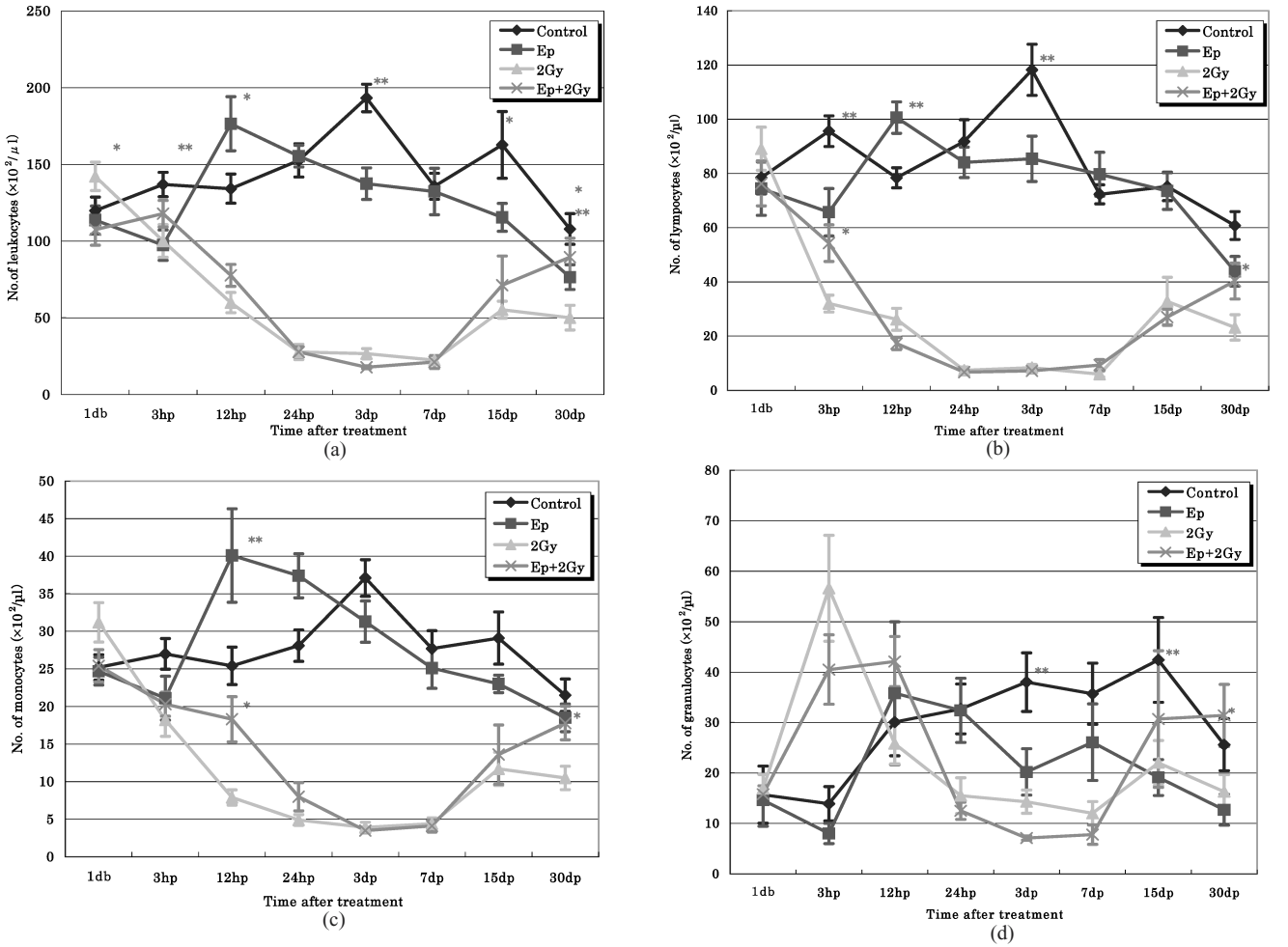

Fig. 1. Each Lineargram Represents the Mean Value±S.E. in (a) Leukocytes, (b) Lympocytes, (c) Monocytes, and (d) Granulocytes from 10 Mice (M)

db, day before treatment; hp, hours posttreatment; dp, days posttreatment. Significantly different $*p<0.05$, $**p<0.01$ control *vs. E. purpurea*, 2Gy *vs. E. purpurea*+2Gy, respectively.

Table 1. Effect of *E. purpurea* on SOD Activity in Mice Serum

Group	SOD activity (%) (mean±S.E.M.)
Control	27.4±2.2
E. purpurea	35.8±2.3*

* Significant difference ($p<0.05$) between control group and *E. purpurea* group, Wilcoxon test.

Table 2. Effect of *E. purpurea* on Total IgG and IgM in Mice Serum

Group	Total IgG (ng/ml) (mean±S.E.)	IgM (ng/ml) (mean±S.E.)
Control	366±26.2	720±62.4
E. purpurea	294±36.4*	600±50.6*

* Significant difference ($p<0.05$) between control group and *E. purpurea* group, Wilcoxon test.

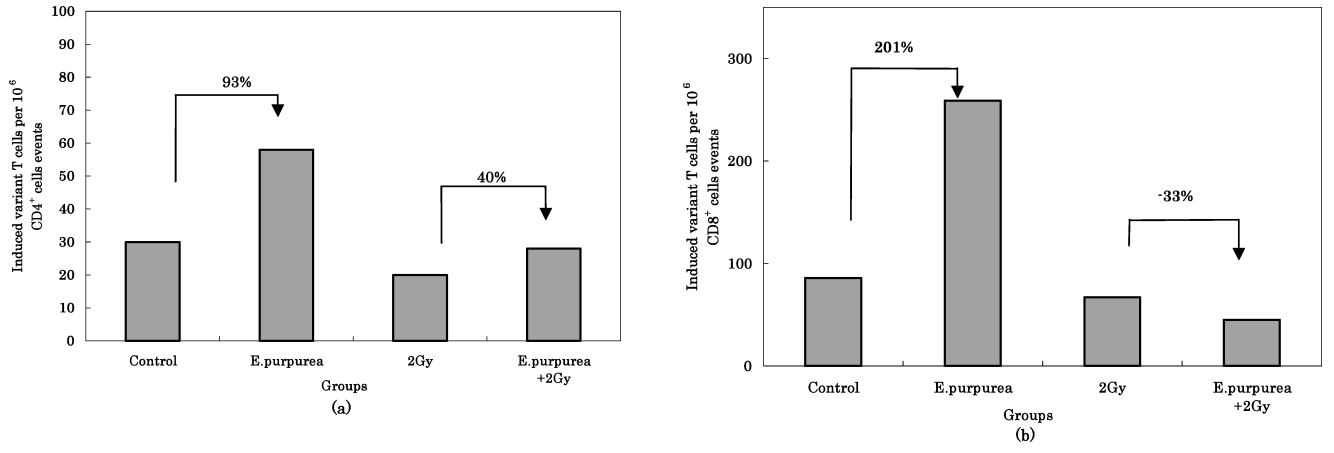

Fig. 2. Comparison of the Induced Frequency of (a) $CD4^+$ and (b) $CD8^+$ in C57BL/6CrSlc Mice

DISCUSSION

Among the consequences of the early response to irradiation, the most problematic is blood cell injury. Usually mature functional cells, such as peripheral blood cells, have low radiosensitivity. However, lymphocytes, although they are mature functional cells, have relatively high radiosensitivity in comparison with other leukocyte types.[12] Therefore in this study, we evaluated the radioprotective effects of *E. purpurea* on blood cells. The study of leukopenia due to irradiation suggested a suppressive effect of *E. purpurea* administration on irradiation-induced leukopenia, especially on lymphocytopenia and monocytopenia. The cause of this effect is likely due to antioxidants in *E. purpurea* such as echinacoside and caffeic acid.[13,14] Hu *et al.* studied the antioxidant effects of *E. purpurea*'s using the DPPH method and reported that Echinacocide and caffeic acid in *E. purpurea* were potent scavengers of free radicals such as hydroxyl radicals ($OH\cdot$) and superoxide (O_2^-)[15] Freeman and Crapo reported that oxidizing-reducing agents reduced the cellular injury caused by O_2^- generated by ionized radiation.[16] The suppressive effect on leukopenia due to radiation in our study also seems to be due to antioxidant substances in *E. purpurea*, such as echinacocide and caffeic acid, which eliminate free radicals generated by irradiation and prevent cellular membrane destruction of blood cells by oxidization.

Goel *et al.* reported that cichoric acid and echinacin in *E. purpurea* activate macrophages.[9] The early recovery in the leukocyte count in our study appears to be due to the ability of polysaccharides and echinacocide to increase the number of leukocytes, and the ability of cichoric acid and echinacin to activate macrophages and to stimulate bone marrow and the reformation of hematopoietic stem cells. In our study, we assumed that SOD activity in peripheral blood was increased because of antioxidants such as echinacocide and caffeine acid in *E. purpurea* which eliminate superoxide (O_2^-) by a free radical scavenging effect. Our results indicate that the suppressive effect of *E. purpurea* on leukopenia due to irradiation in mice is due to an increase in blood antioxidant activity.

The production of IgG and IgM after *E. purpurea* administration was examined. *E. purpurea* administration decreased the production of IgG and IgM in mouse peripheral blood. Rehman *et al.* reported that Echinacea administration for six weeks increased IgG production in the early to middle term in rats.[17] However, in contrast, *E. purpurea* decreased IgG production in the present study. *Echinacea* has interferon (IFN)-like activity to induce and activate macrophages and T lymphocytes, which may explain the difference between the two results.[6] Hayashi *et al.* reported that *Echinacea* sp. increased IFN-γ.[18] Therefore the cell-mediated immune response is activated, while the humoral immune response is in turn suppressed.[18,19] As observed in this study, IFN-like activity of *E. purpurea* stimulated the cell-mediated immune response of macrophages and T cells, while it suppressed the humoral immune response and reduced antibody production.

In this study, CD4-positive T lymphocytes were counted in mouse peripheral blood in each group. *E. purpurea* increased helper T cells in mouse peripheral blood. It seems that *E. purpurea* activated macrophages[6,19,20] and subsequently stimulated T cells. Rininger *et al.*[6] and Burger *et al.*[20] CD-8 positive cells in mouse peripheral blood were examined in each group of mice, and *E. purpurea* increased the number of cytotoxic T cells and suppressor T cells. The mechanism likely involved an increase in INF-γ levels following treatment with *E. purpurea*. The increase in IFN-γ levels, in addition to the suppression of antibody production mentioned previously, activates cell-mediated immune responses, such as proliferation and activation of type I helper T (Th1) cells. CD8-positive cells decreased more in the *E. purpurea* with irradiation group than in the irradiation alone group. Radiation sensitivity increases CD8 cells the most, and the radiation-protection effect of *E. purpurea* depends on dosage.

In conclusion, *E. purpurea* decreased IgG and IgM production by increasing the production of IFN-γ associated with secondary T lymphocyte stimulation by macrophages. In T lymphocyte subsets in mouse peripheral blood, *E. purpurea* administration stimulated macrophages to release cytokines, which in turn stimulated helper T cells to proliferate. Taken together, our results show that *E. purpurea* eliminates free radicals produced by irradiation with its radical-scavenging effect, reduces cytotoxicity such as that caused by lipid peroxides due to oxidization,supresses the decrease in white blood cell counts after irradiation, and prevents the impairment of immunity. It also activates macrophages and T cells and stimulates cytokine production (*e.g.*, IFN-γ), heightens cell-mediated immune responses, prevents the weakening of the immune response associated with irradiation, and presumably bolsters the ability to protect against infection. *E. purpurea* is thus an effective radioprotective agent with low cytotoxicity and few side effects.

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