Food Sci. Biotechnol. Vol. 17, No. 4, pp. 718 ~ 722 (2008)

Food Science and Biotechnology

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Inhibition of Apoptosis by *Elaeocarpus sylvestris* in Mice Following Whole-body Exposure to Ionizing Radiation: Implications for Radioprotectors

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Abstract *Elaeocarpus sylvestris* var. *ellipticus* (E.S.), which contains 1, 2, 3, 4, 6-penta-*O*-galloyl-beta-D-glucose (PGG), is reported to have the ability to scavenge oxygen radicals, thereby protecting rat neuronal cells from oxidative damage. The potential of an E.S. extract, which contains a rich PGG, to protect radiosensitive lymphocytes and intestinal crypt cells from radiation injury induced by a single whole-body irradiation (WBI) *in vivo* was investigated. Our results demonstrated that in immune cells, E.S. treatment decreased the percent of tail DNA, a parameter of DNA damage, compared with levels in untreated, irradiated controls. Furthermore, apoptosis was significantly decreased in lymphocytes and intestinal crypt cells of E.S.-treated mice compared with irradiated controls. These results suggest that the E.S. extract can strengthen the radioresistance of radiosensitive lymphocytes and crypt cells by preventing apoptosis. Therefore, it was concluded that E.S. extract has the radioprotective effects *in vivo* through an inhibition of apoptosis.

Keywords: *Elaeocarpus sylvestris*, lymphocyte, intestinal crypt cell, radioprotection, mouse

Introduction

The free radicals generated by the action of ionizing radiation react with cellular macromolecules, such as DNA, RNA, proteins, membranes, etc, and lead to lipid peroxidation, protein oxidation, base modifications, DNA strand breaks, and genomic instability, ultimately resulting in cell death (1,2). The development of effective radioprotective agents is of great importance in view of their potential application during planned or unplanned radiation exposure. The literature available on radioprotectors is enormous and numerous synthetic drugs, e.g., antioxidants, cytoprotective agents, ACE inhibitors and AT1 receptor antagonists have been tested over the past 50 years (3). Among these radioprotectors, the syntheses of WR-2721, amifostine, ethiophos, and gammaphos, were major breakthroughs in the development of radioprotective drugs (4,5). However, many of these drugs had severe side effects, such as nausea, vomiting, and hypertension (5,6). So the peculiar toxicity of these agents urged researchers to look for safer, less expensive, and more effective radioprotectors.

Natural agents extracted from plants are known to be effective in accelerating recovery from disease or injury and do not appear to have obvious side effects. These nontoxic agents that can protect cells and tissues against ionizing radiation have considerable potential as radioprotective agents (7-10). So, novel bioactive components from plants with substantially improved radioprotective efficacy are needed.

Elaeocarpus sylvestris var. *ellipticus* (E.S.) is a genus of tropical and subtropical evergreen trees and shrubs. Its region of distribution includes the subtropical zone, from Jeju in Korea to southern China, Okinawa, Kyushu (Japan), and Taiwan. E.S., which contains 1, 2, 3, 4, 6-penta-*O*-galloyl-beta-D-glucose (PGG), has been reported to inhibit the expression of inducible nitric oxide synthase and the production of prostaglandin E2 in Raw264.7 cells (11). Furthermore, PGG protected rat neuronal cells from oxidative damage via the induction of neuronal heme oxygenase-1 (HO-1) (12). PGG was also found to be a potent inhibitor of DNA cleavage and an active scavenger of oxygen-free radicals (13). Therefore, we considered an E.S. extract as a likely source of protection from DNA damage caused by γ-rays through its ability to reduce free radicals induced by ionizing radiation.

Exposure to whole-body or significant partial-body irradiation by accident or during cancer therapy causes complex, potentially fatal physiological and morphological changes. In particular, the most sensitive cells to the acute effects of radiation are the most highly replicative cells such as lymphocytes and intestinal crypt cells. At intermediate doses, death is associated with malabsorption, gastrointestinal bleeding, and destruction of gastrointestinal tissue, commonly called gastrointestinal (GI) syndrome (2). At lower doses of radiation, destruction of the lymphoid and hematopoietic systems is the primary cause of a pancytopenic predisposition to infection, septicemia, and death (14). To protect the

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Received September 10, 2007; Revised February 4, 2008;
Accepted February 4, 2008

body from radiation-induced damage, various mechanisms such as free radical scavenging and enhancement of DNA repair in lymphocytes and small intestinal crypt cells are considered important (15). Therefore, it was investigated the potential of an E.S. extract to elicit radioprotective effects by inhibiting DNA damage inducing cyto-/histo-protection in these radiosensitive cells after a single dose of whole-body irradiation (WBI) with γ-rays *in vivo*. In the present study, an E.S. extract increased the cells' radioresistance, facilitating the prevention of radiation-induced damage.

Materials and Methods

Preparation and composition of E.S. extract The E.S. extract was obtained from Dr. Nam Ho Lee (Cheju National University, Jeju, Korea). The E.S. was collected on the Jeju Island of South Korea and the extract was prepared according to a previously described method (16). Analysis of the extract with a nuclear magnetic resonance (NMR) spectrometer revealed characteristic peaks corresponding to galloyl tannins. Subsequent high performance liquid chromatogram (HPLC) revealed that the E.S. extract was rich in PGG (16).

Mice maintenance and treatment with E.S. extract C57BL/6 mice were purchased from Bio Genomics, Inc. (Seongnam, Korea) and were used for survival experiments at the age of 8 to 11 weeks and at body weights (BW) of 20 to 23 g. The animals were housed in conventional animal facilities with a NIH-07 diet and water *ad libitum* at a constant temperature (23±1°C) according to the guidelines for the Care and Use of Laboratory Animals of the Institutional Ethical Committee. For administration as an inoculum, the E.S. extract was dissolved in phosphate-buffered saline (PBS, pH 7.4). To investigate mechanisms that protect animals from radiation-induced damage, the E.S. extract was injected intraperitoneally (i.p.) at a concentration of 10 mg/kg BW at 1 day before irradiation and on the day of irradiation. Control mice were injected with PBS. Mice were randomly divided into the following 4 groups: sham irradiated control, irradiated control, E.S. extract-treated group, and E.S. extract-treated, irradiated group.

Irradiation with ^{60}Co γ-rays A ^{60}Co irradiator (Theratron-780 teletherapy unit; Applied Radiological Science Institute, Cheju National University, Korea) was the source used to irradiate the mice. Briefly, the mice were situated in a close-fitting Perspex box (3×3×11 cm) and received WBI at doses of 2 Gy for experiments. The dose rate was 1.5 Gy/min in the box, and the source-surface distance was 150 cm.

Preparation of primary lymphocytes Briefly, isolated spleens from treated and untreated mice were removed aseptically and placed in sterile Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Paisley, UK). Single cell suspensions were then obtained using a cell strainer. The cells were lysed by adding ammonium chloride potassium carbonate (ACK) buffer, including 0.84% ammonium chloride, and kept at room temperature for 10 min. The cells were then washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco-BRL), and viability was determined by Trypan blue dye (Sigma-Aldrich, St. Louis, MO, USA) exclusion. The lymphocytes (viability >90%) were suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), 1% antibiotics (100 U/mL penicillin-streptomycin, Gibco-BRL), and these purified cells were used directly for experiments.

Alkaline comet assay To assess the extent of DNA damage caused by ionizing radiation, we used the comet assay. Mice were sacrificed 3 days after WBI, their spleens were isolated, and the lymphocytes were placed in suspension containing 1×10^5/mL cells. After washing with DPBS, the cells were mixed with 75 μL of 0.7% low melting point agarose (LMA, Invitrogen, Carlsbad, CA, USA) at 39°C and spread on fully frosted microscopic slides precoated with 1% normal melting point agarose (NMA, Sigma-Aldrich). The slides were kept at 4°C for 10 min to allow the agarose to solidify followed by the addition of a second layer of 100 μL of 0.7% LMA at 37°C. The slides were again placed at 4°C for solidification of the LMA followed by submersion in a chilled lysing solution consisting of 2.5 M NaCl, 100 mM Na_2-ethylene diaminetetraacetic acid (EDTA), 10 mM Tris, pH 10, 1% dimethyl sulfoxide (DMSO), 1% Triton X-100, and 1% sodium sarcosinate for 1 hr at 4°C. The slides were then removed from the lysing solution and placed in a horizontal electrophoresis tank filled with freshly prepared alkaline buffer (300 mM NaOH, 10 mM Na_2-EDTA). After equilibration in the same buffer for 20 min and electrophoresis (25 V, 300 mA), the slides were washed 3 times for 10 min at 4°C in a neutralizing buffer (0.4 M Tris, pH 7.5) and then stained with 45 μL of ethidium bromide (20 μg/mL, Sigma-Altrich). Finally, the slides were observed with a fluorescence microscope and image analyzer (Komet 5.5; Kinetic Imaging, Liverpool, UK), which enabled us to record the tail length, tail movement, and percentage of total fluorescence in the tail of the 50 cells per slide.

4'6-Diamidino-2-phenylindole, dihydrochloride (DAPI) staining To determine the effect of the E.S. extract on the radiation-induced apoptosis of lymphocytes, DAPI staining was performed (17). Mice were sacrificed 1 day after WBI, and spleens were separated and placed in suspension. The cell suspensions were fixed with Carnoy's fixative solution and incubated in 1 μg/mL DAPI solution for 10 min in a dark room. Then the cells were observed with a fluorescence microscope (Leica, Wetzlar, Germany). The frequency of apoptotic cells was calculated as the ratio of apoptotic cells detected to total lymphocytes (500 lymphocytes/mouse) inspected.

Hematoxylin and eosin (H&E) staining To identify the effect of the E.S. extract on the radiation-induced apoptosis of crypt cells in small intestine, H&E staining was performed. Mice were sacrificed 1 day after WBI, and the small intestines were separated and fixed in 10% buffered formalin. The blocks were then embedded in paraplast wax and 5 μm sections were cut and stained with H&E. Crypts were cut longitudinally, and most of the lumen was selected for analysis. Apoptotic crypt cells were counted under a

light microscope. Apoptosis was assessed based on morphological characteristics, such as cell shrinkage, chromatin condensation and margination, and cellular fragmentation as described by Jee *et al.* (18). Five small intestinal sections/mouse and 50 crypt sections/intestine were analyzed.

Statistical analysis Results are reported as means±standard deviation (SD) or standard error mean (SEM). The results were analyzed using ANOVA and the student's *t*-test, and $p<0.05$ was considered significant.

Results and Discussion

E.S. extract reduces the DNA damage in lymphocytes The alkaline comet assay, an effective methodology for monitoring the extent of DNA strand breaks and protection from such breakage (19,20), was used to evaluate the influence of the E.S. extract on ionizing radiation-induced DNA damage of lymphocytes. Augmented cellular DNA damage was measured as an increased percentage of tail DNA, tail movement and tail length. After animals were exposed to 2 Gy γ-radiation, the tail DNA of lymphocytes increased to 41.8±10.9%, compared to the sham irradiated control (9.8±5.4%). Additionally, after exposure to 2 Gy of WBI, the tail movement and tail length increased to 28.7±1.8 and 154.7±5.3 μm, respectively. In contrast, in the sham irradiated control, the tail movement and tail length were 3.7±0.4 and 59.0±6.6 μm, respectively. However, as compared with the irradiated controls, the levels in these parameters decreased to 16.1±5.4% ($p<0.05$), 9.3±1.0 μm ($p<0.01$), and 131.5±10.0 μm in the E.S. extract-treated, irradiated group, respectively (Fig. 1). These results indicated the remarkable cytoprotective effect of E.S. from radiation-induced DNA damage of lymphocytes *in vivo*.

WBI, under the proper conditions, is highly lethal, an effect that is mediated largely through the generation of free radicals, which attack vital cellular sites such as DNA and cell membranes (11). As a result of ionizing radiation, DNA suffers from the breakage of single- as well as double-strands (11), and molecules with the ability to scavenge free radicals can prevent such damage. The alkaline comet assay is an elegant and effective technique for monitoring the extent of DNA strand breaks and protection from such breakage (20-22). When cellular DNA undergoes damage, that process is reflected in an increase of tail length and movement and a rise in the percentage of DNA in the tail, among other factors. By using the comet assay, we observed that the E.S. extract inhibited radiation-induced DNA damage, apparent as a decrease in the percentage of tail DNA, tail movement, and tail length as compared to untreated, irradiated controls.

A number of medicinal plants have proven capacities to provide protection from the damaging effects of ionizing radiation (7,8,11). Extracts from such plants have elicited radioprotective efficacy by virtue of their antioxidative activity (23,24). E.S. extract is rich in bioactive PGG, which is also known for its inhibitory effect on oxidative DNA cleavage by scavenging superoxides and hydroxyl radicals (13).

Similarly, in our study, the E.S. extract inhibited DNA damage from radiation-induced oxidative free radicals.

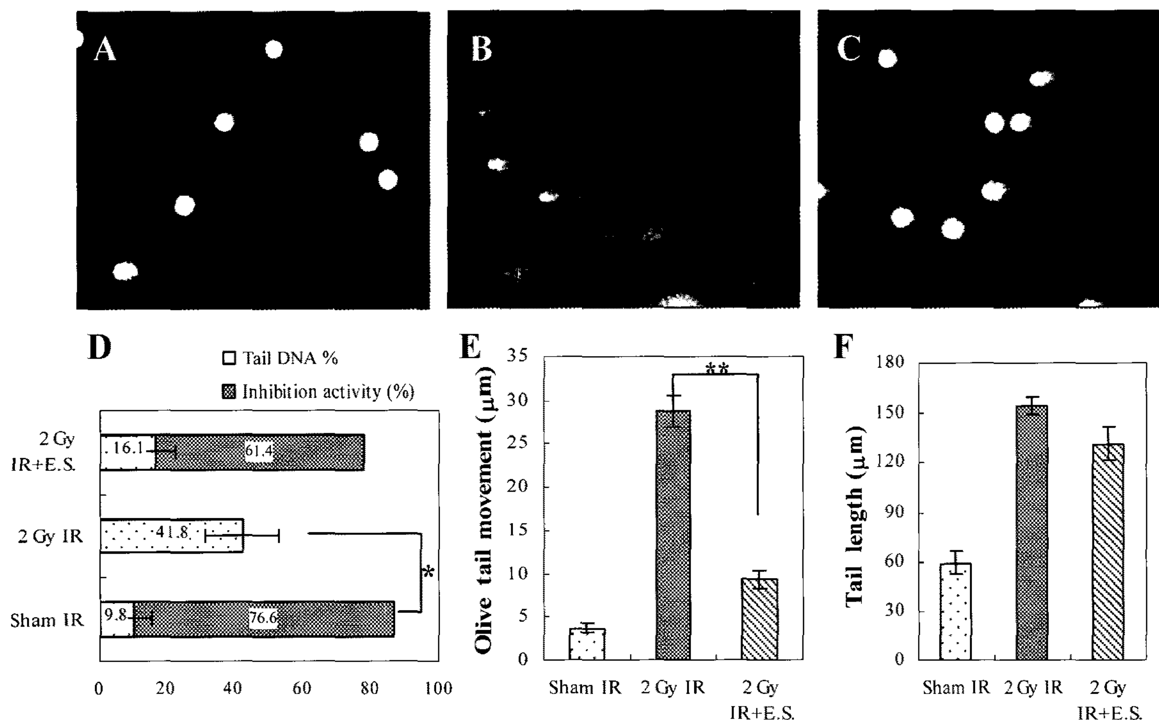

Fig. 1. Comet assay to determine the effect of E.S. extract on irradiation-induced DNA damage in splenocytes comparing. Sham irradiation (A), 2 Gy irradiation (B), 2 Gy irradiation plus E.S. extract (10 mg/kg BW, i.p.) treatment (C). The columns indicate the percentage of tail DNA and of the inhibition activity (D), tail length (E), and tail movement (F) in each group. Each data point represents the mean±SE of 50 cells per mouse. Inhibition activity was evaluated by comparing the 2 Gy irradiation control group to the extract-treated irradiation group by using the Student's *t*-test (*$p<0.05$, **$p<0.01$). The results are representative of 5 separate experiments (n=5).

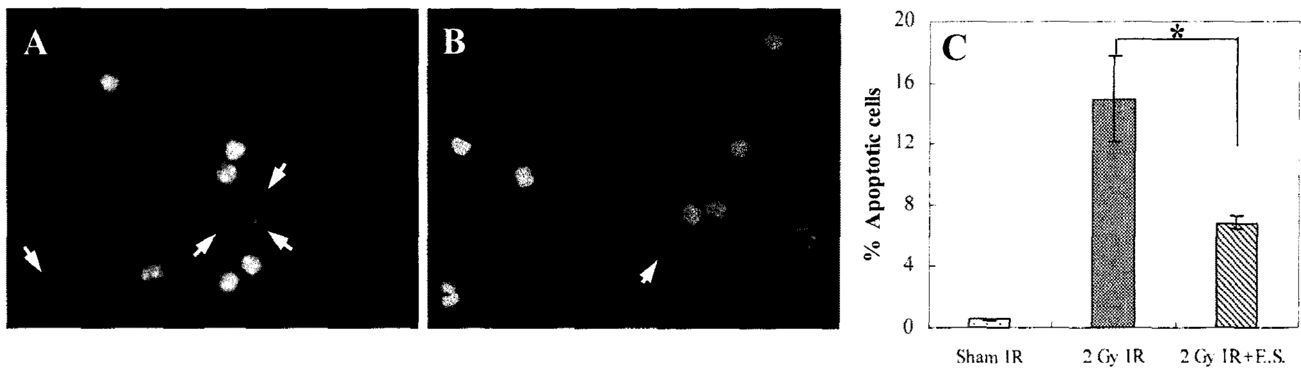

Fig. 2. The effect of E.S. extract on apoptosis of lymphocytes using DAPI staining. Mice were exposed to 2 Gy irradiation alone (A), or 2 Gy irradiation plus E.S. extract (10 mg/kg BW, i.p.) treatment (B). Columns indicate the percentage of apoptotic cells in each group (C). The arrows point to apoptotic cells with condensed and fragmented nuclei. Each data point represents the mean±SE (* $p<0.05$).

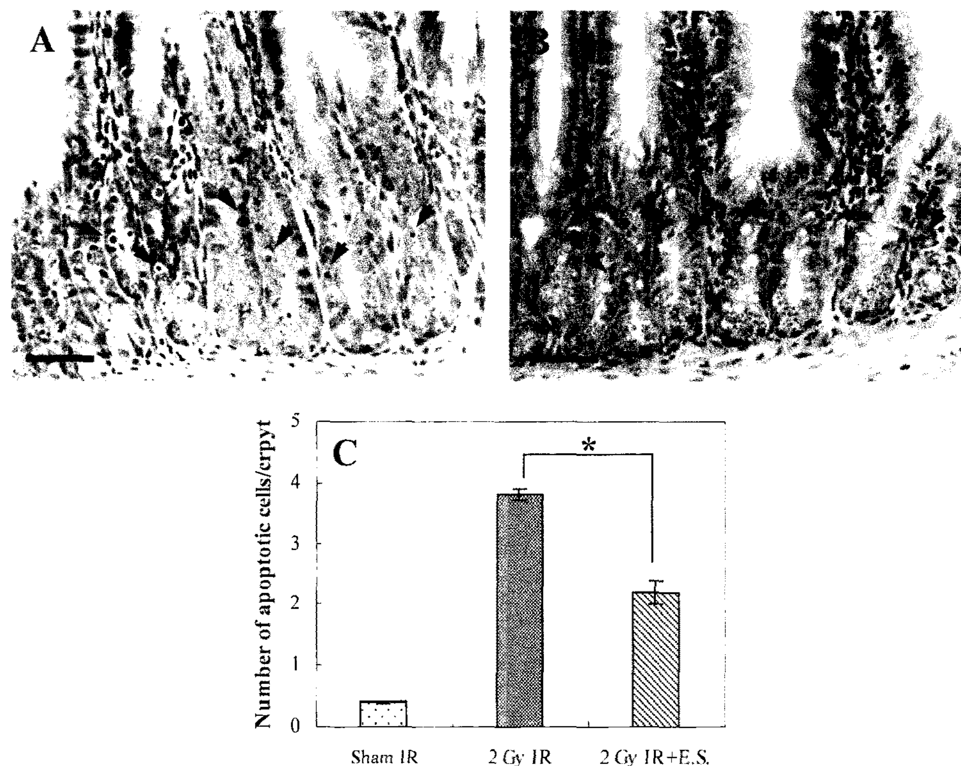

Fig. 3. Morphological classification of typical apoptotic fragments in small intestinal crypt cells using H&E staining. 2 Gy irradiation (A), 2 Gy irradiation plus E.S. extract (10 mg/kg BW, i.p.) treatment (B). Columns indicate the number of apoptotic fragments per small intestinal crypt in each group (C). Values are means±SE of 50 crypts per section of intestine, and 5 small intestinal sections from each mouse were analyzed (* $p<0.05$). Bars=60 μm.

Therefore, the E.S. extract elicits a radioprotective effect via potent radical scavenging activity that eliminates free radicals thereby preventing DNA damage. The PGG component of the E.S. extract, which is an oxygen radical scavenger, may contribute to this protective effect.

E.S. extract inhibits apoptosis in lymphocytes Next, to ascertain the cytoprotective effect of the E.S. extract on radiation-induced apoptosis, we stained the nuclei of lymphocytes with DAPI in preparation for fluorescence microscopy. The resulting images showed that these nuclei were undergoing a significant amount of fragmentation and condensation, indicating the ongoing apoptotic process. In lymphocytes from mice treated with the E.S. extract plus irradiation, the percentage of apoptotic nuclei was dramatically lower than in the untreated, irradiated controls (6.8±0.4 vs. 14.9±2.8% in controls, $p<0.05$, Fig. 2), indicating the protection of cells from radiation-induced death *in vivo*. These finding demonstrate that the E.S. extract could inhibit apoptosis in lymphocytes damaged by ionizing radiation.

E.S. extract decreases apoptosis of intestinal crypt cells We tested small intestinal crypt cells for the E.S. extract's ability to rescue them from radiation-induced apoptosis. The apoptotic fragment assay was used, since intestinal crypt cells are among the rapidly proliferating cell types that are extremely sensitive to irradiation. When the mice

were sacrificed 1 day after WBI, the number of apoptotic fragments per intestinal crypt in the irradiation control group deprived of E.S. extract was 3.8±0.1, whereas that of the irradiated group treated with the E.S. extract was 2.2±0.2, a significant 27.6% reduction ($p<0.05$, Fig. 3). Even these especially radiosensitive intestinal crypt cells exhibited an enhancement of viability due to the E.S. extract inhibition of WBI-induced apoptosis.

Radiation disrupts replicative cells such as lymphocytes and intestinal crypt cells. WBI induces a loss in the number of intestinal crypts and a shortening of the villi (25,26). In both cell types, the fragmentation of chromosomal DNA is the biological hallmark of apoptosis. Here, treatment with the E.S. extract dramatically decreased the radiation-induced fragmentation of nuclei and reduced the apoptosis of lymphocytes and small intestine crypt cells compared with the irradiated control group. This protection of even the most radiosensitive tissues and cells from radiation-induced apoptosis highlights an important mechanism of the E.S. extract's efficacy.

In the present study, we have demonstrated that E.S. extract is an effective radioprotective agent that prevents cell death and promotes recovery from irradiation. Thus, we conclude that E.S contains properties that strengthen the radioresistance of radiosensitive cells and that E.S. extract may be therapeutically beneficial as an effective radioprotective agent without adverse effects.

Acknowledgments

This research was performed under the program of Basic Atomic Energy Research Institute (BAERI) which is a part of the Nuclear R&D Programs funded by the Ministry of Science & Technology (MOST) of Korea. The authors wish to thank P. Minick for editorial assistance.

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