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A comparative study of radioprotection with *Callophyllis japonica* extract and amifostine against lethal whole body gamma irradiation in mice

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SUMMARY

The efficacy of the radioprotective effect of *Callophyllis japonica* ethyl acetate (CJEA) extract was studied by comparing it to that of amifostine, a well-known radioprotective agent, and by evaluating the dose reduction factor, an indicator of radioprotective efficacy. Pretreatment with CJEA extract (100 mg/kg body weight) prior to receiving 12 Gy irradiation significantly improved the survival of jejunal crypts at 3.5 day post-irradiation, but attenuated the level of malondialdehyde compared to vehicle alone ($P < 0.01$). A similar gastroprotective effect was also obtained in the amifostine-treated irradiated group ($P < 0.01$). The efficacy of the radioprotective effect was further confirmed by the dose reduction factor, 1.41. Collectively, these results suggest that CJEA extract is a useful radioprotectant whose efficacy is similar to that of amifostine and whose radioprotective mechanism is in part the reduction of lipid peroxidation caused by gamma irradiation.

Key words: *Callophyllis japonica*; Red seaweed; Jejunum; Radioprotection; Amifostine; Antioxidation

INTRODUCTION

Radiation alters the maintenance of reactive oxygen species (ROS) in various cells and tissues and elevates ROS damage in cellular components and structures, including various proteins, membranes, and nucleic acids, resulting in apoptotic cell death (Miura, 2004). The search for radioprotectors has yet to uncover an efficacious agent in the field of radiation biology (Nair *et al*., 2001).

Amifostine, a synthetic compound, is a selective radioprotector, but it has side effects as well (Schuchter, 1996). Therefore, the search is ongoing for useful radioprotectors with low toxicity and an extended window of protection (Karbownik *et al*., 2000; Nair *et al*., 2001; Hosseinimehr, 2007). Natural products may be potential radioprotectors, as they

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are relatively less toxic than synthetic compounds (Miura, 2004; Hosseinimehr, 2007).

Callophyllis japonica (*C. japonica*), a red seaweed, is abundant in the coastal regions of Jeju Island in South Korea. *C. japonica* extracts shows radical-scavenging activity and lipid peroxidation inhibitory activity *in vitro* (Kang *et al*., 2005) and hepato-protective effects in CCl_4-induced liver injury *in vivo* (Park *et al*., 2005). In radiation experiments, either hexane or ethyl acetate extracts of *C. japonica* increased survival rates (Kim *et al*., 2008). However, the mechanism of its radioprotective efficacy and the corresponding dose reduction factor (DRF) remain to be determined.

The aim of this study was to evaluate the protective effects of one of the more potent radioprotective extracts of *C. japonica*, an ethyl acetate extract (Kim *et al*., 2008). We thus examined the survival of intestinal stem cells and the modulation of lipid peroxidation in the liver in response to irradiation among mice treated with either *C. japonica* ethyl acetate (CJEA) extract or amifostine, another well-known radioprotective agent.

MATERIALS AND METHODS

Animals

Female BALB/c mice (6 - 8 weeks old, n = 50; Orient Bio, Korea) were used in these experiments. All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Jeju National University.

Extraction and compound

CJEA extract was collected as described previously (Kim *et al*., 2008). CJEA was chosen because it had the most potent radioprotective effects among *C. japonica* extracts in a previous study (Kim *et al*., 2008). CJEA extract was dissolved in phosphate-buffered saline and administered to mice (n = 5) intraperitoneally 24 h and 1 h before irradiation (100 mg/kg body weight). Amifostine ([2-(3-aminopropyl) aminoethyl phosphorothioate]; WR2721; Sigma-Aldrich, St. Louis, MO, USA) was administered using this same protocol (n = 5). Control mice (n = 5) were injected with phosphate-buffered saline alone.

Irradiation

Mice were placed in a specially designed, well-ventilated acrylic container and were subjected to whole-body irradiation at 12 Gy (distance = 1.5 m) in a single session using a 60 Co γ-ray source (10,000 Ci; C-188, Canada MDS Nordion; Co-60 Irradiation Facility, Applied Radiological Science Research Institute, Jeju National University, Korea) as described previously (Kim *et al*., 2008).

Intestinal crypt assay

Jejunal crypt stem cell survival was determined using the microcolony technique and was assayed as described previously (Withers and Elkind, 1970; Moon *et al*., 2008). To determine the DRF, we examined mice that had and had not been pretreated with CJEA extract for each irradiation dose point (10, 11, and 12 Gy; 5 mice per dose). Mice were sacrificed 3.5 days after irradiation. The jejunum was fixed in 10% neutral formalin, embedded in paraffin, and cut into slices 5 μm thick. The slides were then stained with hematoxylin and eosin. Two sections of four different parts of the jejunum were prepared for histological examination. The number of regenerating crypts in the jejunal cross-section was then counted. The number of crypts per transverse circumference was counted under a microscope for 10 histological sections per mouse. The average number of crypt cells was plotted against the dose. D10 is the dose at which the curve meets 10 crypt cells. The DRF was determined using the following formula: DRF = D10 with CJEA/D10 with saline.

Malondialdehyde (MDA) assay

The MDA assay was performed as described

previously (Ohkawa *et al*., 1979; Shin *et al*., 2008). Briefly, mice were sacrificed 3.5 days after irradiation, and their livers were homogenized in ice-cold 1.15% KCl. One hundred microliters of the homogenate supernatant was mixed with 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (adjusted to pH 3.5), and 1.5 ml 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4 ml with distilled water and heated to 95 °C for 2 h. After samples had cooled to room temperature, 5 ml of a n-butanol and pyridine mixture (15:1) was added to each sample, and samples were shaken. After centrifugation at 1,000 ×g for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm. The amount of thiobarbituric acid reactive substance was determined using a standard curve with 1,1,3,3-tetrahydroxypropane.

Statistical analysis

Results are presented as mean ± S.E. Data were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls post hoc test for multiple comparisons. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Histologically, there were no pathological changes in the normal controls (Fig. 1A, E). At 3.5 days post-irradiation, the height of jejunal villi had decreased in the vehicle-treated irradiated controls (Fig. 1B, F) compared to the untreated normal controls. Mice that had been pretreated with either amifostine (Fig. 1C, G) or CJEA extract (Fig. 1D, H) had taller jejunal villi 3.5 days after irradiation than vehicle-treated irradiated controls. The histological photos were not shown in this paper because those are similar to the findings in our previous radioprotective experiment (Kim *et al*., 2008; Moon *et al*., 2008).

Table 1 shows the results of the jejunal crypt survival assay. There were significantly fewer jejunal crypts in the vehicle-treated irradiated group (28.95 ± 0.86) than in the normal control group (105.7 ± 1.55, $P < 0.001$). The number of

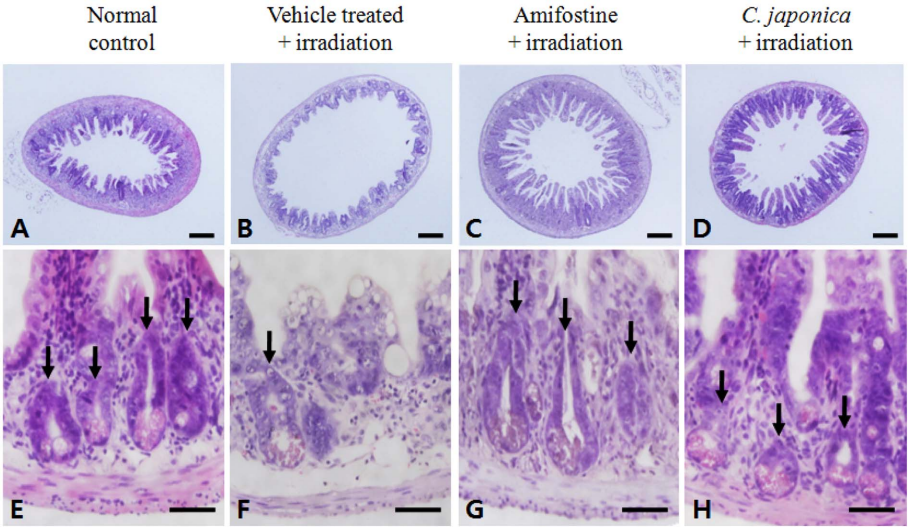

Fig. 1. Representative images showing the villi height (A - D, cross-section of jejunum) and crypt survival (E - H) in the jejunal circumference in the hematoxylin and eosin stained sections from the normal control (A and E), vehicle treated irradiated group (B and F), Amifostine treated irradiated group (C and G) and an ethyl acetate extraction of *C. japonica* treated irradiated group (D and H). The arrows indicate jejunal crypts (E-H). (A-D) scale bars = 100 μm; (E-F) scale bars = 50 μm.

Table 1. Effect of amifostine and *C. japonica* ethyl acetate (CJEA) extract on the survival of intestinal crypts in irradiated mice

Group	Crypts per circumference (Mean ± S.E.)
Normal control	105.7 ± 1.55
Vehicle + Irradiation	28.95 ± 0.86
Amifostine + Irradiation[a]	47.64 ± 8.27[b]
CJEA extract + Irradiation[a]	68.81 ± 2.88[c]

[a]Mice (5 per group) were pretreated intraperitoneally with either amifostine (100 mg/kg) or CJEA extract (100 mg/kg) 24 h and 1 h before gamma irradiation. [b]$P < 0.01$ compared to the vehicle-treated irradiated group. [c]$P < 0.001$ compared to the vehicle-treated irradiated group.

Table 3. Effect of amifostine and *C. japonica* ethyl acetate (CJEA) extract on malondialdehyde levels in the livers of irradiated mice

Group	Lipid peroxidation (nmol/mg) (mean ± S.E.)
Normal untreated control	1731 ± 123
Vehicle + Irradiation	2704 ± 121
Amifostine + Irradiation[a]	1797 ± 109[b]
CJEA extract + Irradiation[a]	1748 ± 83[b]

[a]Mice (5 per group) were pretreated intraperitoneally with either amifostine (100 mg/kg) or CJEA extract (100 mg/kg) 24 h and 1 h before gamma irradiation. [b]$P < 0.001$ compared to the vehicle-treated irradiation group.

jejunal crypts was significantly increased in the CJEA extract group (68.81 ± 2.88) compared to the vehicle-treated irradiated controls ($P < 0.001$).

Furthermore, the number of jejunal crypts was significantly increased in the amifostine-treated mice (47.64 ± 8.27) compared to the vehicle-treated irradiated controls ($P < 0.01$). These findings suggest that CJEA extract has better radioprotective properties than amifostine in intestinal stem cells. Dose reduction factor is an important indicator of radioprotection. As shown in Table 2, the D10 values of mice pretreated with CJEA extract and saline were 13.37 Gy and 9.5 Gy, respectively. The DRF among mice pretreated with CJEA extract who underwent whole-body irradiation was 1.41, suggesting that this extract is radioprotective in this model.

As shown in Table 3, liver MDA levels were significantly higher in the vehicle-treated irradiated group (2704 ± 121 nmol/mg) than in normal untreated controls (1731 ± 123 nmol/mg; $P < 0.001$). MDA levels were significantly reduced in the amifostine-treated group (1797 ± 109 nmol/mg) compared to the vehicle-treated irradiated group (2704 ± 121 nmol/mg; $P < 0.001$). This suggests that amifostine treatment reduces lipid peroxidation in irradiated mice. MDA levels were significantly lower in mice treated with CJEA extract (1748 ± 83 nmol/mg) than in the vehicle-treated irradiated group (2704 ± 121 nmol/mg; $P < 0.001$). No differences in MDA levels were observed between the amifostine and CJEA extract groups.

DISCUSSION

Previously, we reported that hexane and ethyl acetate extracts of *C. japonica* protect against radiation-induced mortality by reducing the number of bone marrow nucleated cells (Kim *et al.*, 2008). In this study, we examined whether the administration of CJEA extract to mice prior to undergoing whole-body irradiation improved survival of jejunal crypts and protected against radiation-induced oxidative stress in the liver. We found that the radioprotective efficacy of CJEA extract was similar

Table 2. Regression analysis of the intestinal crypt assay

Group	Intercept (b)	Slope (m)	y = mx + b	D10	DRF
Vehicle + Irradiation	200	-20	10 = –20x + 200	9.50	1.41
CJEA + Irradiation	200.75	-14.265	10 = –14.265x + 200.75	13.37	

DRF, dose reduction factor; CJEA, ethyl acetate extract of *C. japonica* (100 mg/kg).

to or better than that of amifostine, a well-known radioprotective agent.

Proliferating stem or progenitor cells are particularly vulnerable to radiation. Because small intestinal crypts contain so many proliferative stem cells, they have been used to evaluate the in vivo radioprotective effects of both chemical compounds and natural products (Sigdestad *et al*., 1976; Potten and Grant, 1998; Goel *et al*., 2003; Kim *et al*., 2008; Moon *et al*., 2008, 2009). In this study, we examined damage to jejunal crypts to determine the extent of the in vivo radioprotective effect of CJEA extract. CJEA extract improved the survival rate of jejunal crypts 3.5 days after irradiation. Furthermore, this radioprotective effect was demonstrated by the DRF found among mice pretreated with CJEA extract (DRF = 1.41).

Whole-body irradiation is lethal, and its effects are mediated mainly through cellular damage, including DNA damage, lipid peroxidation, and protein oxidation (El-Missiry *et al*., 2007; Moon *et al*., 2009). In addition, radiation-induced ROS causes cellular oxidative damage by initiating lipid peroxidation. Lipid peroxidation is a marker of lipid damage and is commonly measured by determining the level of thiobarbituric acid reactive substance that includes MDA and 4-hydroxyalkenals (Karbownik *et al*., 2000). This study shows that MDA levels in the liver after irradiation are reduced by pretreatment with CJEA extract. A similar result was also obtained for amifostine pretreatment. A methanolic extract of *C. japonica* exhibits scavenging activity toward intracellular ROS and 1,1-diphenyl-2-picrylhydrazyl radicals. It promotes cell viability, inhibits H_2O_2 production, inhibits apoptosis, and enhances the effects of antioxidant enzymes (Kang *et al*., 2005). Thus, we suggest that treatment with CJEA extract prior to irradiation ameliorates lipid peroxidation in the liver by inhibiting radiation-induced ROS.

In conclusion, CJEA extracts might be useful radioprotectors of intestinal progenitor cells, possibly through the suppression of lipid peroxidation caused by gamma irradiation.

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