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The radioprotective effects of the hexane and ethyl acetate extracts of *Callophyllis japonica* in mice that undergo whole body irradiation

Jeongtae Kim[1,†], Changjong Moon[6,†], Heechul Kim[1], Jinwoo Jeong[1], Juyeon Lee[1], Jihoon Kim[1], Jin Won Hyun[2,3], Jae Woo Park[2,4], Mi Yeon Moon[5], Nam Ho Lee[2,5], Sung Ho Kim[6], Youngheun Jee[1,2,3], Taekyun Shin[1,2,3,*]

[1]College of Veterinary Medicine, and the Research Institute for Subtropical Agriculture and Biotechnology, [2]Applied Radiological Science Research Institute, [3]Department of Biochemistry, College of Medicine, [4]Department of Nuclear and Energy Engineering, College of Engineering, [5]Department of Chemistry, College of Natural Science, Cheju National University, Jeju 690-756, Korea
[6]Department of Veterinary Anatomy, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea

The radioprotective activity of extracts from the red seaweed *Callophyllis* (*C.*) *japonica* was investigated in mice that underwent whole-body exposure to gamma radiation. A methanol extract of *C. japonica* and its fractions [hexane, ethyl acetate (EtOAc), butanol and the remaining H_2O] were used. Each fraction (100 mg/kg body weight) was administered intraperitoneally (i.p.) 2 times into the BALB/c mice, once at 1 and once at 24 h before exposure to 9 Gray (Gy) of gamma radiation. Pre-irradiation administration of the hexane and EtOAc fractions saved the mice, with their survival rates being greater than 80% at 30 days post-irradiation; the mice that were pretreated with the other fractions showed survival rates lower than 20% over the same time period. To examine the effect of each *C. japonica* fraction on the survival of intestinal and bone marrow stem cells, the number of intestinal crypts and bone marrow cells in the gamma-irradiated mice were examined. Pre-treatment of mice (i.p., 100 mg/kg body weight at 1 and 24 h before irradiation) with the hexane or EtOAc fraction prior to 6-Gy irradiation significantly protected the number of jejunal crypts and bone marrow cells at 9 days after irradiation. These findings suggest that certain extracts from *C. japonica*, when they are administered prior to irradiation, play an important role in the survival of irradiated mice, and this is possibly due to the extracts protecting the hematopoietic cells and intestinal stem cells against gamma irradiation.

Keywords: bone marrow cells, *Callophyllis japonica*, mice, radioprotection

*Corresponding author
Tel: +82-64-754-3363; Fax: +82-64-756-3354
E-mail: shint@cheju.ac.kr
[†]The first and second authors contributed equally to this work.

Introduction

Radiation causes various pathophysiological alterations in living animals, and it causes death at high doses by multiple mechanisms, including direct DNA damage and indirect oxidative stress [4,7]. The search for useful radioprotectors has been an important issue in the field of radiation biology [9]. Ideal radioprotectors should have low toxicity and an extended window of protection [2,4]. As many synthetic compounds have toxic side effects, the natural products have attracted scientific attention as radioprotectors. Natural products that have been recently shown to be effective radioprotectors were found to have anti-oxidant and immunostimulant activities [2,3,8,12]. Thus, antioxidant and immunostimulant activities may play roles in protection against irradiation damage.

The red seaweed *Callophyllis* (*C.*) *japonica* is abundant in the coastal regions of Jeju Island in South Korea. A previous *in vitro* study showed that *C. japonica* extracts exhibit intracellular reactive oxygen species, 1,1-diphenyl -2-picrylhydrazyl radical-scavenging activity and lipid peroxidation inhibitory activity [3]. Furthermore, *C. japonica* has been demonstrated to be cytoprotective in CCl_4-induced liver injury [10], and an ethanol extract of *C. japonica* has been shown to have protective effects on radiation-induced intestinal injury [6]. However, there's not much known about the *in vivo* radioprotective effects of *C. japonica*. This study investigated the effects of *C. japonica* on mice that were exposed to a sub-lethal dose of gamma radiation.

Materials and Methods

Fractionation of *C. japonica*

The *C. japonica* was collected during August 2006 from

Jeju Island in Korea and the collected seaweed was identified by a taxonomist, Dr. Y.P. Lee, Cheju National University, Korea. The air-dried leaves of *C. japonica* (1,100 g) were powdered and extracted with 80% methanol (MeOH; Merck, Germany) at 95°C. The extract was filtered, evaporated to dryness under reduced pressure and then concentrated in vacuo. The lyophilized crude MeOH extract (65 g) was successively extracted with n-hexane (hexane; Junsei Chemical, Japan), ethyl acetate (EtOAc; Junsei Chemical, Japan), and n-butanol (BuOH; Junsei Chemical, Japan), to obtain the hexane (0.62 g), EtOAc (1.13 g), BuOH (1.5 g), and remaining water (H_2O, 48.06 g) fractions; the extraction yields were 1%, 1.7%, 2.3% and 73.9% (w/w), respectively (Fig. 1).

Animals and experiments

Female BALB/c mice (6-8 weeks old; Orient Bio, Korea) were used in these experiments. Each extract from *C. japonica* was dissolved in phosphate-buffered saline and administered intraperitoneally at 24 and 1 h before irradiation (100 mg/kg body weight). After treatment, the mice were placed in a specially designed, well-ventilated acrylic container and they were subjected to whole-body irradiation at 6 or 9 Gray (Gy) in a single session with using a ^{60}Co Y-ray source (10,000 Ci; C-188, Canada MDS Nordion; Co-60 Irradiation Facility, Applied Radiological Science Research Institute, Cheju National University, Korea), as was described in our previous reports [1,5,6]. All the experiments were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals at Cheju National University, Korea.

Survival assays

Survival was monitored daily and this was reported as the percentage of animals that were still alive at 30 days after 9-Gy irradiation. The mice used for this study were divided into five groups: irradiation plus vehicle (control) and four treatment groups, one for each *C. japonica* extract.

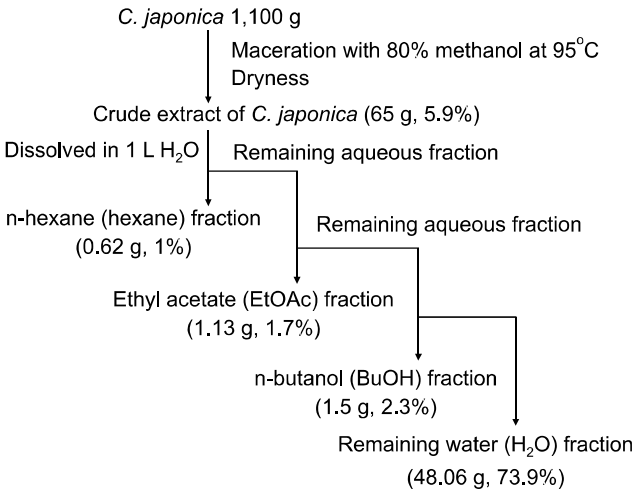

Fig. 1. Flow diagram of the fractionation of a crude extract from *Callophyllis japonica*.

Determination of the number of bone marrow cells

To test the effect of *C. japonica*, the number of bone marrow cells was counted 9 days after the mice were irradiation with 6 Gy. Each treatment group consisted of five mice. Bone marrow cells were obtained from the anesthetized mice by aseptic isolation of the femurs, from which the marrow was flushed with Hank's balanced salt solution (HBSS; Invitrogen, USA) and using a 25-gauge needle. The cells were suspended in HBSS and then they were counted using a hemocytometer. The results are expressed as the number of live bone marrow cells ($\times 10^6$) /femur.

Jejunal crypt assay

The jejunal crypt stem cell survival was determined with using the microcolony technique described by Withers and Elkind [14]. Each treatment group consisted of five mice. The mice were sacrificed 9 days after their irradiation (6 Gy). Two sections of four different parts of the jejunum from each animal were prepared for histological examination. The regenerating crypts in the jejunal cross-sections were then counted.

Statistical analysis

The results are presented as mean ± SE. The results were compared between each extract group and the vehicle-treated controls by using Student's unpaired, one-tailed t-test. In all cases, p values < 0.05 were deemed to be statistically significant.

Results

Survival rate of the mice after irradiation

For the control mice that were given 9 Gy irradiation, 80% died by day 12, and all of them died before 15 days post-irradiation (Fig. 2). The mortality rate of the irradiated mice that were pre-treated with the remaining water-soluble extract was 60% at day 12 and 100% by day 15.

For the mice pre-treated with the BuOH, EtOAc, and hexane fractions prior to irradiation, 20, 80 and 100%, respectively, of the animals were alive at day 30. The mortality rates for the irradiated mice that were treated with the hexane and EtOAc extracts were significantly reduced compared with the mortality rate for the control group. These results suggest that the hexane and EtOAc fractions of *C. japonica* effectively decreased the radiation-induced mortality.

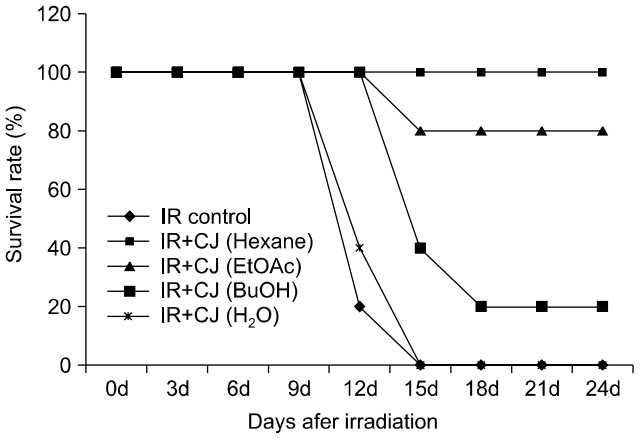

Fig. 2. The effect of each extract of *Callophyllis japonica* (CJ) on the survival of mice exposed to irradiation (9 Gy). Each extract was administered intraperitoneally twice, once at 24 and once at 1 h before irradiation (IR). The data is expressed as percentage of surviving mice.

Effect of the *C. japonica* extracts on bone marrow nucleated cells

The number of bone marrow cells (Fig. 3) was significantly lower in the irradiation-only group ($2.22 \pm 0.31 \times 10^6$ cells /femur) than that in the normal control group ($13.28 \pm 1 \times 10^6$ cells/femur, $p < 0.05$). The numbers of bone marrow cells in the irradiation groups that had been pre-treated with the hexane and EtOAc extracts ($6.06 \pm 0.77 \times 10^6$ and $4.11 \pm 0.74 \times 10^6$ cells/femur, respectively) were significantly higher than the number of bone marrow cells in the irradiation-only group ($p < 0.05$ for each extract). There was no significant protective effect on the numbers of bone marrow cells in the groups that were treated with the BuOH and remaining water-soluble fractions ($2.93 \pm 1.89 \times 10^6$ and $1.23 \pm 0.27 \times 10^6$ cells/femur, respectively) compared with that of the irradiation-only group.

Effect of the *C. japonica* extracts on the survival of intestinal crypts

Fig. 4 shows the results of the jejunal crypt survival assay. The number of jejunal crypts was significantly lower in the irradiation-only group (81.25 ± 2.53) compared with the normal control group (104.57 ± 5.32, $p < 0.05$). The number of jejunal crypts in the hexane extract-treated mice with irradiation (88.36 ± 2.48) and in the EtOAc extract-treated group (101.13 ± 1.6) was significantly increased compared with the number of jejunal crypts in the irradiation-only controls ($p < 0.05$ for each extract). The number of jejunal crypts in mice that were pre-treated with the BuOH extract (78.55 ± 5.78) or the remaining water-soluble fraction (77.26 ± 2.96) was not significantly different from the number of jejunal crypts in the irradiation-only control group.

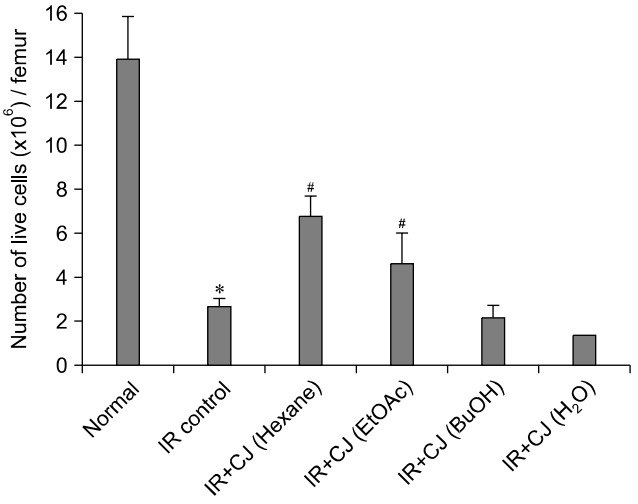

Fig. 3. The effect of each extract of *Callophyllis japonica* (CJ) on the bone marrow cellularity in the radiation-treated mice. Pre-treatment with the hexane and EtOAc extracts increased the bone marrow cellularity as compared with that in the irradiation (IR)-only group. Each *C. japonica* extract was administered intraperitoneally twice, once at 24 and once 1 h before irradiation. Hematopoietic stem cell assays were performed 9 days after gamma-irradiation of 6 Gy. The data is expressed as the mean ± SE. $^*p < 0.05$ compared with the normal controls; $^\#p < 0.05$ compared with the irradiation-only group.

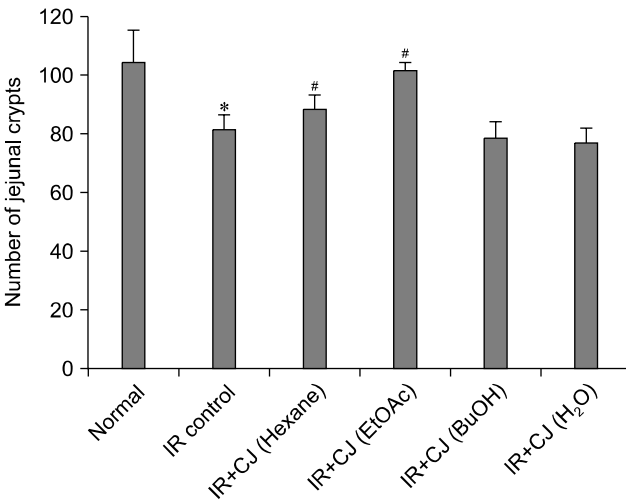

Fig. 4. The effect of each extract of *Callophyllis japonica* (CJ) on intestinal crypt survival in the radiation-treated mice. Pre-treatment with the hexane and EtOAc extracts increased the number of jejunal crypts as compared with the number of jejunal crypts in the irradiation (IR)-only group. Each *C. japonica* extract was administered intraperitoneally twice, once at 24 and once 1 h before irradiation. The jejunal crypt assays were performed 9 days after 6-Gy irradiation. The data is expressed as the mean ± SE. $^*p < 0.05$ compared with the normal controls; $^\#p < 0.05$ compared with the irradiation-only group.

Discussion

Our study indicates that certain extracted fractions of *C. japonica* (the hexane and EtOAc fractions) provided protection against radiation-induced mortality. Moreover, our data shows that administration of the hexane or EtOAc fractions of *C. japonica* prior to irradiation reduced the decrease of bone marrow nucleated cells that was induced by radiation. The death of the irradiated animals was largely attributable to hematopoietic syndrome, which is characterized by a impaired bone marrow hematopoietic function, and this leads to leukopenia, erythropenia and thrombocytopenia [15]. Thus, administration of *C. japonica* reduced the mice's radiation-induced mortality, and it apparently did so by protecting the blood progenitor cells from the effects of irradiation.

The number of intestinal crypts is generally accepted as a good indicator of the protection of intestinal stem cells and/or the proliferation of surviving cells in animals that are recovering from exposure to radiation [8]. Stem cells in crypts of the small intestine are particularly vulnerable to radiation because of their high rate of proliferation [11,13]. The enhanced number of intestinal crypts in the *C. japonica*-treated/irradiated mice indicates that the *C. japonica* extracts protected the stem cells or the extracts stimulated the proliferation of the surviving cells. Thus, we suggest that treatment with *C. japonica* extract prior to irradiation protected the intestinal stem cells, and so this resulted in an increased survival rate.

The effects of *C. japonica* extracts in whole-body irradiated animals are not fully understood, but one possible mechanism involves their antioxidant properties. An extract of *C. japonica* exhibited scavenging activity toward intracellular reactive oxygen species and 1,1-diphenyl-2-picrylhydrazyl radicals, it promoted cell viability, inhibited H_2O_2 production, inhibited apoptosis and enhanced the effects of antioxidant enzymes [3]. The molecular and cellular mechanisms for the radioprotective effects of *C. japonica* extracts remain to be determined.

In conclusion, our results suggest that the administration of *C. japonica* extract to mice prior to irradiation increased their survival rate and this increased survival rate was associated with the protection of hematopoietic and intestinal stem cells.

Acknowledgments

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