

Article

Protective Effects of Delphinidin against Proton Beam Induced Damage in Human Colon-Derived CCD-18Co Cells

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Abstract: Unavoidable exposure to radiosensitive normal tissues around cancerous tumor during the radiotherapy can cause side effects such as self-limited acute toxicities, mild chronic symptoms, or severe organ dysfunction. Nevertheless, clinical use of currently available radiation protective agents is limited because of their generic cytotoxicity. A study on radiation protective effect of delphinidin was conducted with proton-beam-exposed human colon cells (CCD-18Co). The measurement in changes of survival fractions of CCD-18Co with/without delphinidin administration at different radiation doses were measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The changes in expression of reactive oxygen species (ROS) and in activities of antioxidant enzymes were measured by colorimetric assays using pertinent assay kits. The measurement of pro-apoptosis/pro-survival protein expressions using Western blot assay and the measurement of DNA damage using comet assay were also fulfilled to evaluate the molecular level of radiation damages in CCD-18Co cells. The experimental results revealed that the pre-administration of delphinidin regulated antioxidant enzymes, reduced ROS, decreased DNA damage, regulated pro-apoptosis/pro-survival proteins, and eventually reduced apoptosis of CCD-18Co cells. In conclusion, it is claimed that delphinidin is nontoxic natural radiation protective compound, and thus delphinidin can be used to protect normal colon tissues during the proton beam therapy.

Keywords: delphinidin, radiation protective agent, proton beam therapy, CCD-18Co cells, reactive oxygen species, antioxidant enzyme, DNA damage

1. Introduction

Radiotherapy is one of the most common treatments for cancer patient, and the radiation is frequently used in conjunction with chemotherapy or with surgery [1]. The primary goal of radiotherapy is to destroy cancer cells by delivering curative radiation energy to the cancer cells with tolerable damages to normal tissues. However, when the radiation travels through a patient's body during the therapeutic exposure, it interacts with not only cancer cells but also normal tissue cells located at ahead / behind of the cancerous tumor along the penetrating passage. The exposure to the normal cells happens always during the radiation exposure even with the sophisticated dose delivery systems such as conformal radiotherapy, intensity-modulated radiotherapy, and image-guided radiotherapy [2]. In other words, a certain amount of radiation exposure to the normal tissues around a cancerous tumor is inevitable result of radiation therapy. Figure 1 shows the relative depth dose distribution. The contents of normal tissue would be corresponding to highly radiosensitive tissue or critical organs around the tumor and the normal tissue margins with microscopic disease. The exposures to those normal tissues may cause various side effects such as self-limited acute toxicities, mild chronic symptoms, or severe organ dysfunction [2].

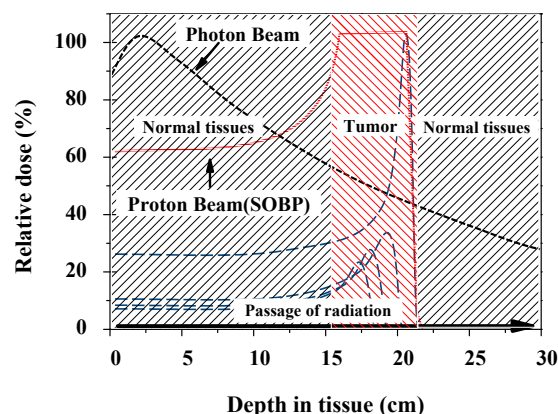


Figure 1. Relative depth dose distribution of photon beam (black dashed line) and proton beam (blue dashed line) in tissue. The Bragg peak can be spread out to encompass the tumor (SOBP; Spread out Bragg peak, red solid line).

Colorectal cancer is the third most common cancer in Korea, and the rate of incidence ranks the first top through the world. According to the announcement of National Cancer Information Center, the incidence of colon cancer accounts for 12.4% of all cases. The number of death from colon cancer in 2015 was 8,380, accounting for 10.9% of all deaths from cancer. Surgery is the most common treatment for the patients with early-stage colon and rectal cancer, but chemo-radiotherapy is the main treatment for the patients with advanced colon and rectal cancer [1]. However, human colon tissue are very sensitive to radiation, so exposure of normal colon tissue during the radiotherapy may leads to the side effect such as diarrhea, bowel dysfunction, and radiation enteritis [3,4]. According to Crystal S. Denlinger, etc., they reported that patients receiving colorectal radiotherapy suffered from discomfort in activities of daily living and social activities up to 5 years after treatment due to clustering of bowel movements (42%), increase in nighttime bowel movements (46%), incontinence (39%), pad wearing (41%), and inability to defer defecation (78%) [3]. Nevertheless, since the exposure to normal tissue is unavoidable, the protection or remedy of normal tissues from the severe damage by the administration of radiation protective agent prior to/simultaneously with the radiation exposure must be considered.

The most of currently available agents including amifostine has limit in clinical use because they induces severe adverse effects such as nausea, vomiting, hypotension, nephron and neuro-toxicity [5,6]. Therefore, the study to develop the more effective and the safer radioprotective agents with less side effects is necessary. Anthocyanins are one of the flavonoid subtypes. They are phytopigment substances in the fruit, flower, stem, and leaf of a plant that give off bluish purple, black, and purple. Delphinidin, one of the major anthocyanidins, is the strongest antioxidative phytochemical compound due to the greatest number of hydroxyl groups in the B-ring of its chemical structure (Figure 2) [7]. It is reported that delphinidin antioxidative property is endowed by the mechanisms such as hydrogen atom transfer (HAT), single electron transfer (SET) [8].

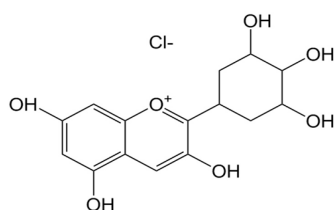


Figure 2. Chemical structure of delphinidin.

75
76 Though the radioprotective property of delphinidin against photon exposure has been verified
77 by researchers [9-11], the studies against proton beams have not been conducted yet. Proton beam is
78 a higher linear energy transfer (LET) radiation than photon beam such as X-rays and gamma-ray. In
79 general, radiation protective agents are known to be effective against low LET radiation but not
80 against high LET radiation. However, it was revealed by Alan Mitteer et al. that proton beam
81 produced much more reactive oxygen species (ROS) than the same dose of photon beam did [12].
82 Therefore, the present study was conducted to investigate the radiation protective effect of
83 delphinidin against proton beam induced damage in human colon-derived CCD-18Co cells.

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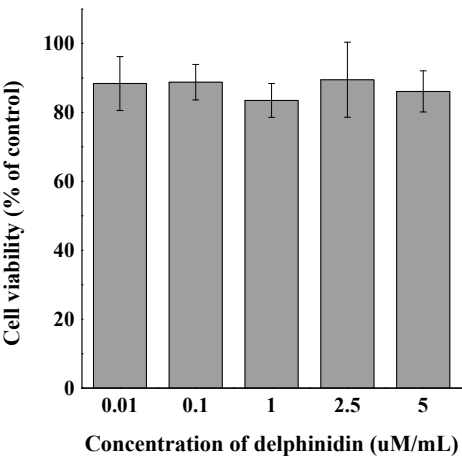
85 **2. Results**

86 We performed the following experiments to evaluate the radioprotective effect of delphinidin; MTT
87 assay, SOD activity assay, CAT activity assay, DCF-DA assay, comet assay, western blot assay in
88 CCD-18Co cells.

89

90 *2.1. Cytotoxicity of delphinidin*

91 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was performed to
92 evaluate the cytotoxicity of delphinidin due to the various concentrations from 0.01 μ M to 5 μ M in
93 CCD-18Co cell lines, and the results were shown in Figure 3. There were no significant differences in
94 cytotoxicity in colon cell with the concentrations less than 5 μ M of delphinidin. It was confirmed that
95 delphinidin did not affect cell viability of the cell lines at the concentrations ranging from 0.01 μ M to
96 5 μ M. Therefore, we determined the concentrations of delphinidin for the proton beam irradiation
97 experiment; Low delphinidin group: 2.5 μ M, High delphinidin group: 5 μ M.



98 **Figure 3.** Cytotoxicity of delphinidin in CCD-18Co cells. The cells were treated with the
99 concentration of 0.01 ~ 5 μ M/ml delphinidin in culture medium for 24 h. The cell viabilities at the
100 various concentrations were determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium
101 bromide (MTT) assay. Results were expressed as the percentages of control by means \pm standard
102 deviations of eight independent experiments.

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2.2. Effects of delphinidin on the viabilities of CCD-18Co cells

Cell survival assays were performed to proton-exposed CCD-18Co cell lines to estimate radio-protective effect of delphinidin at various radiation doses. As demonstrated in Figure 4, survival fraction of the cell lines decreased as the radiation dose increased. Also, the survival fractions of both groups treated by delphinidin (EG-LDp and EG-HDp), EG-Dp, were higher than that of EG at all dose. The survival fractions of EG-LDp and EG-HDp showed significant differences from EG at 1Gy ($p < 0.01$). The experimental data was summarized in table 1.

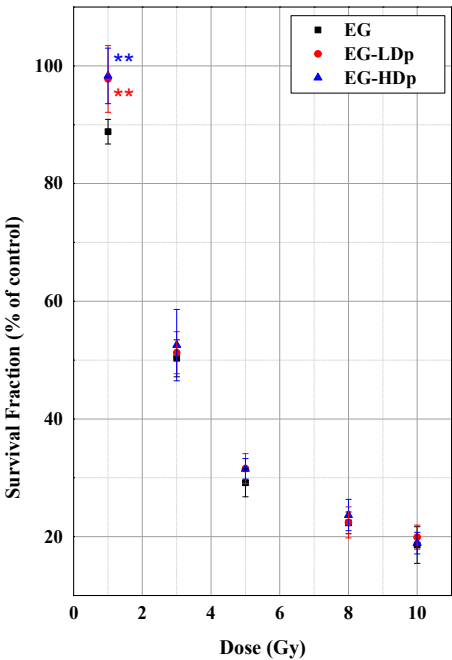


Figure 4. Effects of delphinidin on the viabilities of the proton-exposed CCD-18Co cells. The cells were treated with 2.5 μ M or 5 μ M of delphinidin for 24 h prior to irradiation and were exposed to different doses (1, 3, 5, 8 and 10 Gy). The measured survival fractions are mean \pm standard deviations of eight independent experiments. Statistical significance was evaluated by the one-way analysis of variance (ANOVA). The ** symbols stand for $p < 0.01$, compared with EG.

Table 1. Effects of delphinidin on the viabilities of the proton beam-exposed CCD-18Co cells.

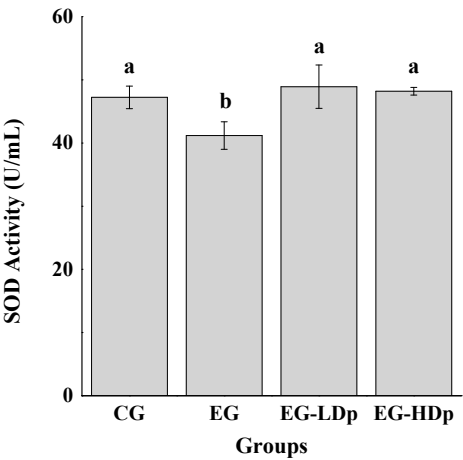
Groups	1 Gy	3 Gy	5 Gy	8 Gy	10 Gy
EG	88.82±2.09 ¹⁾	50.32±3.14	29.16±2.38	22.33±1.85	18.58±3.10
EG-LDp	97.79±5.67 **	51.27±3.58	31.56±2.56	22.42±2.64	19.91±2.06
EG-HDp	98.30±4.72 **	52.55±6.06	31.53±1.73	23.67±2.66	18.91±1.84

¹⁾ Mean \pm S.D., ** $p < 0.01$ vs. EG

2.3. Effects of delphinidin on Superoxide dismutase (SOD) activity

SOD activity assays were performed to estimate antioxidant activity of delphinidin against proton beam-induced oxidative stress in CCD-18Co cell lines. As demonstrated in Figure 5, radiation exposure decreased the activity of SOD compared to CG (CG: 47.23 ± 1.78 , EG: $41.18 \pm$

124 2.17). However, the activity of SOD in EG-Dp was increased compared to that of EG. The EG-LDp
125 and EG-HDp showed differences from EG with statistical significance ($p < 0.05$).
126



127 **Figure 5.** Effects of delphinidin on Superoxide dismutase (SOD) activity in proton-exposed
128 CCD-18Co cells. The measured data were expressed as mean \pm standard deviations of four
129 independent experiments. Statistical significance was evaluated by the one-way analysis of variance
130 (ANOVA).

131

132 2.4. Effects of delphinidin on Catalase (CAT) activity

133 CAT activity assays were also performed to estimate antioxidant activity of delphinidin
134 against proton beam-induced oxidative stress in CCD-18Co cell lines. The results were shown in
135 Figure 6. The activity of CAT in EG was significantly decreased compared to CG. (CG: 1.23 ± 0.31 ,
136 EG: 0.14 ± 0.09). On the other hand, the activity of CAT in EG-Dp did not show significant
137 difference compared to that of EG. However, the activity of CAT reduced by radiation tended to
138 slightly increase again by pre-treatment of delphinidin.

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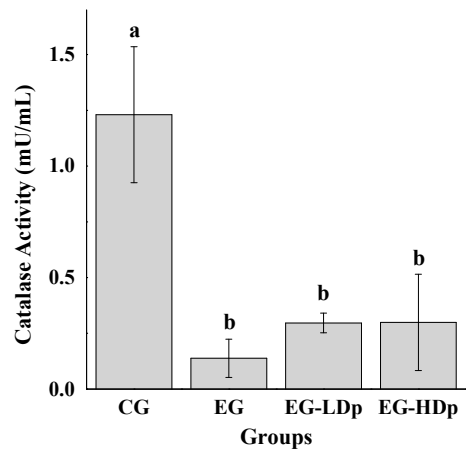


Figure 6. Effects of delphinidin on Catalase (CAT) activity in proton-exposed CCD-18Co cells. The measured data were expressed as the mean \pm standard deviations of four independent experiments. Statistical significance was evaluated by the one-way analysis of variance (ANOVA).

2.5. ROS scavenger effects of delphinidin

DCF-DA assays were performed to evaluate ROS scavenging activity of delphinidin against proton beam-induced ROS in CCD-18Co cell lines. As demonstrated in Figure 7, radiation exposure significantly increased the amount of ROS expression compared to CG (CG: 61765.8 ± 6767.5 , EG: 108360.6 ± 27220.23). Whereas, the amount of ROS expression in EG-Dp was significantly decreased compared to EG ($P < 0.05$).

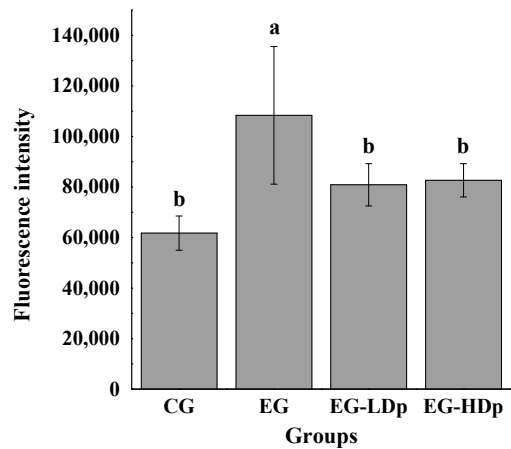
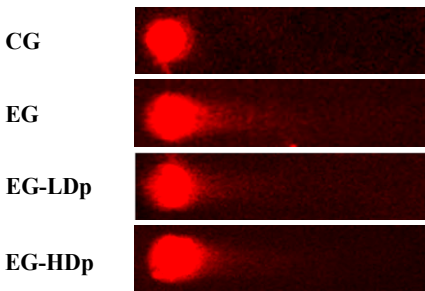


Figure 7. Reactive oxygen species (ROS) scavenger effects of delphinidin in proton-exposed CCD-18Co cells. ROS scavenger effect of delphinidin was measured by the DCF-DA assay. Results are expressed as intensity of DCF-DA fluorescence. The results are mean \pm standard deviations of eight independent experiments. Statistical significance was evaluated by the one-way analysis of variance (ANOVA).

156 2.6. Protective effects of delphinidin on the DNA damage

157 Comet assays were performed to evaluate the radio-protective effect of delphinidin against
158 proton beam-induced DNA damage. Results of comet assay were shown in Figure 8. Tail was not
159 observed in CG of CCD-18Co cells. The tail length and the tail intensity of EG were measured the
160 longer and the higher than those of CG. The length and the intensity of tail in comet assay are
161 proportional to the amount of DNA damage. The lengths and intensities of tails of delphinidin
162 treated groups (EG-LDp and EG-HDp) were the shorter and the lower than those of EG.

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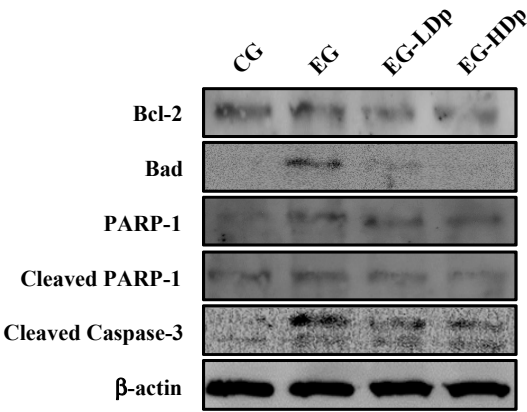


164 **Figure 8.** Effects of delphinidin on the DNA damage in proton-exposed CCD-18Co cells. DNA
165 damage was measured by the comet assay. The cells were stained with EtBr, and the images were
166 photographed by a confocal microscope.

167

168 2.7. Effects of delphinidin on the apoptosis related-protein expression

169 Signal proteins were quantified by western blot assay to evaluate the protective effect of
170 delphinidin against proton beam-induced cell apoptosis in CCD-18Co cells. The pro-survival
171 regulator Bcl-2 and pro-apoptotic proteins such as Bad, PARP-1, cleaved PARP-1 and cleaved
172 caspased-3 were measured. As it was shown in Figure 9, decreased expression of Bcl-2 and
173 increased expression of pro-apoptotic proteins were observed in EG. The expressions of Bad and
174 cleaved caspased-3 were significantly increased compared to CG. Whereas, in EG-LDp and
175 EG-HDp, decreased expression of pro-apoptotic proteins were observed compared to EG. There
176 was no difference between EG-LDp and EG-HDp.



177 **Figure 9.** Effects of delphinidin on the protein expression of Bcl-2, Bad, PARP-1, cleaved PARP-1 and
178 cleaved caspase-3 in proton-exposed CCD-18Co cells. Whole cell lysates were prepared, and
179 analyzed by western blotting using the specific antibodies Bcl-2, Bad, PARP-1, cleaved PARP-1 and

cleaved caspase-3. Equal loading of protein was confirmed by stripping the blot and reprobing with β -actin antibody. Data shown here are from a representative experiment repeated three times with similar results. The experiment was repeated three times with similar results and data here show a representative experiment result.

3. Discussion

The results of survival fraction measurement showed that delphinidin was radioprotective for colon cells without regardless of concentrations only at low dose of proton beam exposure. At the higher radiation doses there were no significant differences in survival fractions between delphinidin treated groups (EG-LDp and EG-HDp) and non-treated groups (EG). This result reflected that delphinidin was radioprotective at the lower radiation doses but was not radioprotective anymore at the higher radiation dose than 3 Gy. According to the results, delphinidin could be used as radioprotective agents for radiation therapy to protect normal tissues which are unintentionally exposed by low dose of radiation around cancerous tumor. In addition, the 50% lethal dose (LD_{50}) was measured to be 3 Gy from the survival fraction measurement. Hence, 3 Gy was used as the reference radiation dose in the molecular biological experiments to measure the activities of enzymes, expression of ROS, changes in protein expression, and DNA damage.

It was found at EG that the exposure of proton beam increased the ROS expression but decreased the activity of SOD and CAT. Ionizing radiation produces large amounts of ROS in nature by the interactions between radiation and cellular molecules. The overexpression of ROS lead to oxidative stress on essential macromolecules such as DNA, protein, and cell membrane consisted of lipid [13,14]. Under normal conditions, the antioxidant defense system, including SOD and CAT, protects the essential macromolecules from oxidative stress. SOD catalyzes the decomposition of toxic $O_2^{\cdot-}$ which is converting $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2) and oxygen gas (O_2). CAT then catalyzes the reaction which is converting $2 \cdot H_2O_2$ to $2 \cdot H_2O$ and O_2 [15]. Therefore, as it was shown in Fig. 5 and 6, the reduced activities of SOD and CAT after irradiation were thought to be due to the increased utilization of antioxidant enzymes to eliminate ROS produced by radiation exposure [9].

On the other hand, it was found at both EG-LDp and EG-HDp that the administration of delphinidin prior to the radiation exposure was helpful for regulating the normal level of ROS expression and the activities of SOD and CAT even with the radiation effects. The upregulation in activities of SOD and CAT was considered as the result of preventing the depletion of SOD and CAT by the ROS scavenger effect of delphinidin [16]. In other words, pre-treatment of delphinidin reduced radiation-induced ROS expression, and sequentially changed the activities of antioxidant enzymes. The results were consistent with those of previous studies. [9,16,17].

Radiation exposure cause cellular damage by direct interactions or indirect interactions between radiation and intracellular molecules. Among the types of radiation-induced cellular damages, DNA damage is the most lethal to the cell viability. According to the results of comet assay as shown in Fig. 7, significant DNA damage was observed in EG, but significantly reduced damage were observed in EG-LDp and EG-HDp. These results combined with the results of ROS expression proved that proton beam exposure provided considerable impact on DNA damage, whereas pre-treatment of delphinidin reduced the damage by scavenging radiation-induced ROS.

In general, the proteins relevant to apoptosis needed to be additionally analyzed for the clarifying of protective mechanism of an agent on ROS-induced DNA damage. According to the results of the measurements of pro-survival/ pro-apoptosis proteins as shown in Fig. 8, proton beam exposure increased the expression of pro-apoptosis protein, whereas the pre-treatment of delphinidin reduced the expression of pro-apoptosis proteins such as Bad, cleaved PARP-1, and cleaved caspase-3. The dephosphorylation of Bad inactivating Bcl-2 and Bcl-xL, and thus allowing Bax/Bak-triggered apoptosis. Whereas, phosphorylation of Bad by Akt/protein kinase B forms the

Bad-(14-3-3)protein heterodimer, and it leaves Bcl-2 free to inhibit Bax-triggered apoptosis. [18] Hence, it could be said that delphinidin effectively reduced dephosphorylation of Bad by scavenging radiation-induced ROS, and thus showed the inhibition of apoptosis.

In conclusion, it was confirmed by the results of the molecular biological experiments that the delphinidin could protect a human normal colon cells from the damages regardless of the concentration at low dose of proton beam exposure. Moreover, since there was no significant difference in protective effect at high dose of radiation, delphinidin was regarded as the safe natural radiation protective agent which would be used for the radiotherapy to protect the normal tissues around the cancerous tumor.

4. Materials and Methods

4.1. Chemicals and Reagents

Delphinidin chloride was purchased from Sigma-Aldrich (St. Louis, Mo., USA) and diluted to less than 1% DMSO and medium.

4.2. Human tissue cells

The cells were purchased from the Korean Culture Type Collection (KCTC; Korea) and maintained in a 5% CO₂ incubator at 37°C. CCD-18Co cells (normal human colon cells) were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS).

4.3. Irradiation

Proton beam exposure was performed at room temperature by the 45 MeV MC50 cyclotron with an average dose rate of 16.8 Gy/min at Korea Institute of Radiological and Medical Sciences (KIRAMS). Sample cells were exposed by the proton beams with spread-out Bragg peak (SOBP).

4.4. Experimental design

The experiments were conducted in four sample groups as follows:

1. Control group (CG): cells with no treatment.
2. Exposed group without pretreatment (EG): cells with 3 Gy exposure of proton beam.
3. Exposed group with low concentration of delphinidin (EG-LDp): cultured cells with 2.5 µM of delphinidin for 24 h and exposed to 3 Gy of proton beam.
4. Exposed group with high concentration of delphinidin (EG-HDp): cultured cells with 5 µM of delphinidin for 24 h and exposed to 3 Gy of proton beam.

4.5. MTT assay

The cell viabilities of the experimental groups were determined by the MTT assay. CCD-18Co cells were seeded at 1×10^4 cells per well in a 96-well plate and incubated for 24 hours at 37°C in a CO₂ incubator. The cells were treated with delphinidin at the concentration of 0.01~5 µM for 24 hours. The cells were treated with 10 µl MTT solution in the dark for 4 h at 37°C. The formazan crystals formed within the cells were dissolved out using 100 µl DMSO, and the absorbance at 540 nm was measured by a microplate reader.

273 4.6. Cell survival assays

274 The cells were seeded in a 96 well plate and incubated for one day at 37°C in a CO₂ incubator.
275 The cells were treated with 2.5 µM or 5 µM of delphinidin for 24 h and were exposed to various
276 doses (1, 3, 5, 8 and 10 Gy). After irradiation, the media was replaced with the new media and
277 incubated for seven day at 37°C in a CO₂ incubator. Then the cells were treated with 10 µl MTT
278 solutions in the dark for 4 h at 37°C. The absorbance was measured by the same process as
279 described in MTT assay.

281 4.7. SOD activity assay

282 The cells lysed with lysis buffer (ice-cold 0.1 M Tris/HCl, pH 7.4 containing 0.5% triton X-100, 5
283 mM β-ME and 0.1 mg/mL PMSF). The lysed cells were centrifuged at 14,000 × g for 5 min at 4°C,
284 and the supernatants were harvested. The SOD activity was evaluated using a SOD activity assay
285 kit (BioVision Inc. Mountain View, CA, USA) in accordance with the manufacturer's instruction.
286 The inhibition activity of SOD was determined by a colorimetric method, and the absorbance was
287 read at 450 nm using a microplate reader.

289 4.8. CAT activity assay

290 The cells were lysed in cold assay buffer and centrifuged at 10,000 × g for 15 min at 4°C. The
291 supernatants were harvested and put into the wells. CAT activity was determined by using
292 Catalase Activity Colorimetric assay kit (BioVision) in accordance with the manufacturer's
293 instruction. In the assay, the activity of CAT was measured at 570 nm using a microplate reader.

295 4.9. Measurement of ROS

296 The cells were treated with 2.5 µM or 5 µM of delphinidin for 24 hours. Then 10 µM of
297 2'-7'-dichlorodihydrofluorescein diacetate (DCF-DA) was added to the culture media prior to
298 irradiation. The cells were immediately exposed to 3 Gy proton beam and incubated for 30 min.
299 After 30 min, the cells were washed three times in PBS. The plate was read at excitation wavelength
300 485 nm and emission wavelength 535 nm using fluorescence activated cell sorter (FACS).

302 4.10. Comet assay

303 After irradiation, the cells were carefully detached with 1X trypsin and centrifuged at 1,000
304 rpm for 3 min. The cells were resuspended in 0.5 % low-melting agarose, spread on a pre-coated
305 slides, covered with a coverslip and placed at 4°C for 2 min. The coverslip was removed, and the
306 slide was overnighted into lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 10% DMSO, 1%
307 Triton X-100, pH 10) at 4°C. After lysis, the slides were submersed for 30 min in a chamber
308 containing running buffer (300 mM NaOH, 1 mM EDTA, pH 13) and electrophoresed at 25 V/cm for
309 30 min. Following electrophoresis, the slides were neutralized in 0.4 M tris (pH 7.5) 3 times for 5
310 min and dried at room temperature. The DNA was stained by 0.8 µl/ml ethidium bromide (EtBr).

312 4.11. Western blot assay

313 The cells were exposed to 3 Gy of proton beam and lysed in a RIPA lysis buffer. Lysates were
314 centrifuged at 14,000 rpm for 30 min, and the supernatant was collected. The protein content was
315 determined by use of the Bio-Rad protein assay (Bio-Rad laboratories, Hercules CA). 25 µl of
316 cellular proteins was loaded into each well of a 10 % SDS-PAGE gel and transferred to membrane.
317 The membrane was incubated overnight with primary antibodies against Bcl-2, Bad, PARP-1,

cleaved PARP-1 and cleaved caspase-3, followed by the sequential incubation with secondary antibodies. The blots were detected by enhanced chemiluminescence (ECL).

4.12. Statistical analysis

All measured data were expressed as mean \pm S.D. Statistical significance was evaluated by the one-way analysis of variance (ANOVA), and the differences were considered significant with the condition of $P < 0.05$.

Author Contributions: H.M., S.H.¹ and B.S. conceived and designed the experiments; H.M. and S.H.¹ performed the experiments; H.M., S.H.¹ and S.H.² analyzed the data; H.M. and B.S. wrote the paper.

Conflicts of Interest: "The authors declare no conflict of interest."

Abbreviations

ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
CAT	Catalase
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
HAT	Hydrogen atom transfer
SET	Single electron transfer

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