Food Sci. Biotechnol. 25(S): 163-168 (2016)
DOI 10.1007/s10068-016-0114-7

Food Science and Biotechnology

Radioprotective Effect of Hesperetin against γ-Irradiation-induced DNA Damage and Immune Dysfunction in Murine Splenocytes

Jung Ae Kang[1], Seon Hye Yoon[2], Jong Kook Rho[3], Beom-su Jang[1,4], Dae Seong Choi[1], Dong-Eun Lee[1], Eui-Baek Byun[1], Jongho Jeon[1], and Sang Hyun Park[1,4,*]

[1]*Division of Biotechnology, Korea Atomic Energy Research Institute, Jeongeup, Jeonbuk 56212, Korea*
[2]*Nutrition and Functional Food Research Team, National Institute of Food and Drug Safety Evaluation, Osong, Chungbuk 28159, Korea*
[3]*Center for Neuro-Medicine, Brain Science Institute, Korea Institute of Science and Technology, Seoul 02792, Korea*
[4]*Department of Radiation Biotechnology and Applied Radioisotope Science, Korea University of Science and Technology, Daejeon 34113, Korea*

Received December 11, 2015
Revised February 26, 2016
Accepted March 22, 2016
Published online March 31, 2016

*Corresponding Author
Tel: +82-63-570-3370
Fax: +82-63-570-3371
E-mail: parksh@kaeri.re.kr

pISSN 1226-7708
eISSN 2092-6456

© KoSFoST and Springer 2016

Abstract This study was conducted to evaluate the preventive effect of hesperetin against radiation-induced DNA damage and immune dysfunction in murine splenocytes. Isolated splenocytes from BALB/c mice were treated with hesperetin (20, 100, and 500 μM), and then irradiated at a dose of 2 and 4 Gy of γ-irradiation. Exposure to γ-radiation resulted in DNA damage and a reduction of cell viability as well as an elevation of the levels of proinflammatory cytokines, intracellular ROS (reactive oxygen species), and NO (nitric oxide). Hesperetin significantly enhanced the cell viability of the splenocytes compared with the irradiated group. In addition, hesperetin was found to be highly effective in preventing DNA damage as identified by comet and DNA ladder assays. Hesperetin also effectively inhibited proinflammatory cytokines, intracellular ROS, and NO in irradiated splenocytes. In conclusion, hesperetin was shown to be radioprotective against irradiation-induced DNA damage and immune dysfunction in murine splenocytes.

Keywords: Hesperetin, γ-irradiation, murine splenocytes, DNA damage, immune dysfunction

Introduction

Humans are constantly exposed to ionizing radiation through a variety of sources, including space travel, radiotherapy, working in nuclear plants, and potential accidents (1). Recently, large amounts of artificial radionuclides such as radioiodine and radiocesium were released by the Fukushima Daiichi nuclear power station (FDNPS) accident in March 2011 (2,3). Therefore, people are highly concerned about radiation causing human defects and accordingly, interested in the human defense system as well. The absorption of ionizing radiation by living cells can directly attack the DNA or indirectly generate reactive oxygen species (ROS). In addition, the reaction of ROS with DNA causes DNA damage, apoptosis, and dysfunction of the immune systems (4,5). Radioprotective agents are compounds that possess the ability to protect against the deleterious effects of ionizing radiation. However, synthetic compounds or cytokines have seen limited application as radioprotectors because they are toxic and cause side effects including decreased cellular function, nausea, hypotension, and death in mammalian systems (6). The development of effective, inexpensive, and non-toxic radioprotective agents are now an active area of research (7). Flavonoids are generally recommended as potential radioprotectors because of their high antioxidant activity and scavenging free radicals generated by the radiolysis of water, thereby protecting cells from damage (8).

Flavanone hesperetin (3',5,7-trihydroxy-4methoxyflavanone) is an aglycone of hesperidin, which is the major flavonoid present in oranges and lemons. It has been reported that hesperetin exerts a wide range of biological effects including antioxidant, antiinflammatory, antifungal, antiviral, anticarcinogenic and ultraviolet protective activities (9,10). Dietary hesperidin is deglycosylated to hesperetin by intestinal bacteria prior to absorption (11); thus, hesperidin may be considered as prodrug that is metabolized into hesperetin (12). Hesperidin has been reported to exert protective effects against γ-irradiation-induced hepatocellular damage and oxidative stress in rats (13). There are no reports available on the effect of hesperetin against γ-irradiation-induced damage through *in vitro* studies.

In the present study, murine splenocytes were used they are radiosensitive and organized secondary lymphoid organs (14) and we investigated the preventive effects of hesperetin against γ-irradiation-induced DNA damage and immune dysfunction in murine splenocytes.

Materials and Methods

Chemicals All chemicals, hesperetin, hesperidin, phosphate buffered saline, DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate), and Griess Reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, and streptomycin-penicillin were purchased from Thermo Fisher Scientific (Waltham, MS, USA). Hesperetin and hesperidin were dissolved in dimethyl sulfoxide (DMSO, 0.01%).

Preparation of murine splenocytes Spleens removed from six-week-old female BALB/c (*n*=10) were grinded into small pieces and passed through sterilized cell strainer (Falcon cell strainer; BD bioscience, San Jose, CA, USA) containing RPMI 1640 medium to obtain a homogeneous cell suspension. The murine splenocytes were centrifuged and red blood cells were removed using a hemolytic red blood cell lysis solution. The remaining murine splenocytes were washed twice in PBS. The murine splenocyte suspensions (1×10^6 cell/well) were plated in 96-well tissue culture plates maintained in RPMI 1640 complete medium containing 10% fetal bovine serum and 1% streptomycin-penicilline and then cultured at 37°C in a 5% CO_2 incubator.

Irradiation In all *in vitro* experiments, the cells were pretreated with hesperetin and hesperidin for 2 h in a RPMI 1640 medium before irradiation. The murine splenocytes were exposed to γ-irradiation using a Gammacell 40 Exactor (Nordion Inc., Ottawa, Canada). The cells were exposed to 2 and 4 Gy at a dose rate of 1.1 Gy/min (15).

Cell viability Premix WST-1 (water soluble tetrazolium salt) cell viability assay (TaKaRa Bio Inc., Kusatsu, Japan) is based on a reduction of tetrazolium salt to water soluble formazan by mitochondrial dehydrogenase in viable cells (16). The amount of the formazan dye is directly proportional to the number of living cells. The murine splenocytes were incubated for 24 h at 37°C in 5% CO_2, treated with hesperetin and hesperidin at 20, 100, and 500 μM for 1 h, and then irradiated at 2 or 4 Gy. Twenty four hours later, 10 μL of WST-1 was added to each well, and cells were incubated for 1 h at 37°C in 5% CO_2. The absorbance was measured at 440 nm with a microplate reader (SpectraMax Plus 384 Microplate Reader; Molecular Devices, Sunnyvale, CA, USA).

Intracellular ROS measurement The DCFH-DA method was used to monitor the levels of intracellular ROS. DCFH-DA penetrates into the cells, where it is hydrolyzed by intracellular esterase into DCFH. The DCFH is oxidized by intracellular oxidants into a highly fluorescent, 2',7'-dichlorodihydrofluorescein (DCF) (17). The murine splenocytes were incubated for 24 h at 37°C in 5% CO_2, treated with hesperetin and hesperidin at 500 μM for 1 h, and then irradiated at 2 Gy. Twenty four hours later, 25 μM of DCF-DA was added, and the fluorescence of DCF was detected using a microplate reader.

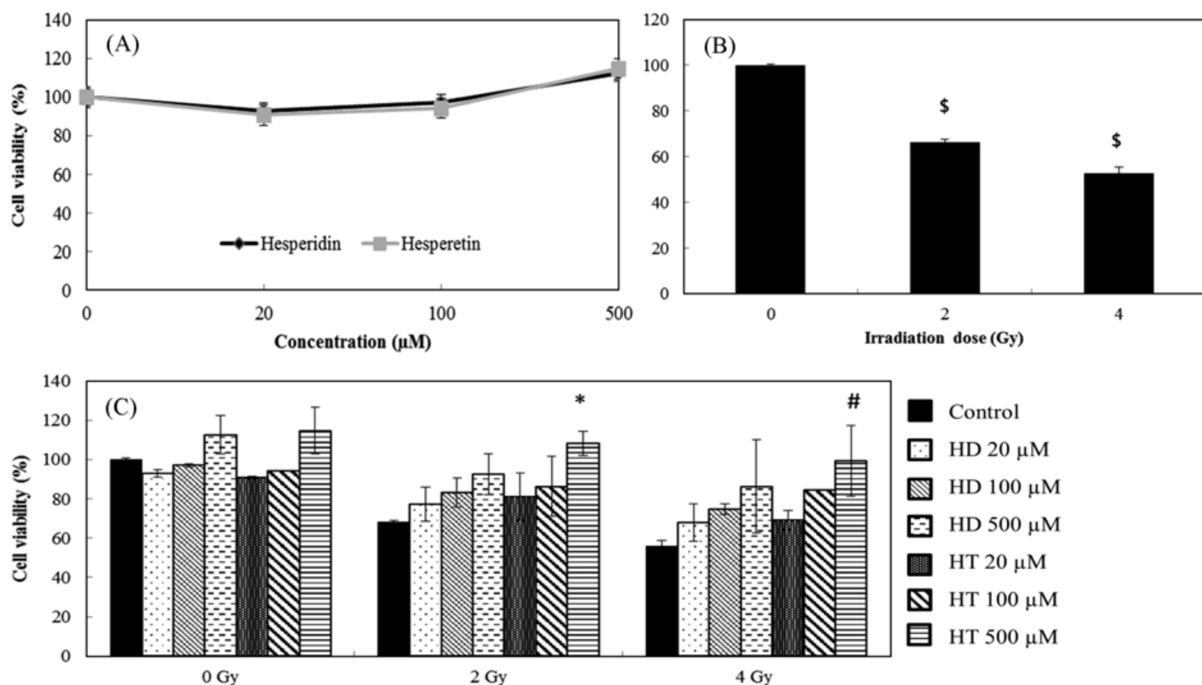

Fig. 1. Effect of hesperetin and hesperidin on cell viability. Cell viability of splenocytes treated with various concentrations of hesperetin and hesperidin (A). Dose dependent inhibition of viability from splenocytes irradiated with different γ-irradiation dose (B). Values are expressed as mean±SD of three experiments. $^{\$}p<0.05$ represents significant differences compared with untreated group. Effect of hesperetin and hesperidin on radiation-induced cytotoxicity in splenocytes (C). Values are expressed as mean±SD of three experiments. $^{*}p<0.05$ represents significant differences compared with 2 Gy of irradiation group. $^{\#}p<0.05$ represents significant differences compared with 4 Gy of irradiation group.

Nitric oxide (NO) productions assay NO levels were determined using a Griess Reagent, a common experimental measure for assessing nitrite, a metabolic byproduct of nitric oxide. For determining the effect of hesperetin and hesperidin, cells were treated with hesperetin and hesperidin at 500 μM for 1 h and then γ-irradiated at 2 Gy. Twenty four hours later, an equal volume of cultured murine splenocytes supernatant and Griess Reagent (100 μL) were mixed. The absorbance was measured at 595 nm using a microplate reader.

Comet assay The comet assay was performed using a OxiSelect Comet Assay Kit (Cell Biolabs Inc., San Diego, CA, USA) according to the manufacturer's instruction. The murine splenocytes were incubated for 1 h with hesperetin and hesperidin at 500 μM of hesperidin and hesperetin and then irradiated (2 Gy). The cells were further incubated for 24 h at 37°C in 5% CO_2. Cells were harvested, washed with PBS and a 10 μL cell suspension containing $1x10^4$ cell mixed with 100 μL of 0.5% agarose at 37°C, and 75 μL of this mixture was instantly added to comet slides. Embedded cells were immersed in a lysis buffer at 4°C for 1 h and then replaced with a cold alkaline buffer and placed at 4°C for 30 min followed by electrophoresis (35 V at 4°C for 15 min) in a horizontal electrophoresis chamber filled with an alkaline electrophoresis buffer. The slides were washed cold distilled water and then placed in 70% ethanol for 5 min. After RedSafe staining for 15 min at room temperature, comets were observed by fluorescent microscopy (Leica DM2500; Leica Microsystems, Wetzlar, Germany). The comet tail lengths were measured in individual embryos using Komet 5.5 (Kinetic Imaging Ltd., Liverpool, UK).

DNA ladder The murine splenocytes were incubated for 1 h with hesperetin and hesperidin (500 μM) and then irradiated (2 Gy). The cells were further incubated for 24 h at 37°C in 5% CO_2. DNA was extracted from murine splenocytes using a DNA extraction kit (Promega, Fitchburg, WI, USA). The cells were collected and washed with PBS. The pellet was re-suspended in 600 μL of lysis buffer containing 3 μL of RNase and incubated at 37°C for 30 min. The mixture was added to protein precipitation (200 μL) and centrifuged at 13,000x*g* for 5 min. DNA was extracted with 600 μL of isopropanol and precipitated with 600 μL of 70% ethanol. DNA integrity was assessed using agarose gel (1.5%) electrophoresis and RedSafe staining (iNtRON Biotechnology, Seongnam, Korea).

Cytokine assay Cells were treated with hesperetin and hesperidin at 500 μM for 1 h and then γ-irradiated at 2 Gy. The supernatant cultured for 24 h were used for the cytokine production test. ELISA kits (BD Bioscience) were used by following the manufacturer's instructions and tumor necrosis factor-α (TNF-α), and IL-6 was evaluated at 450 nm using a microplate reader.

Statistical analysis Statistical analyses were performed using a one-way analysis of variance (ANOVA), and inter group comparisons were made using Tukey's multiple comparison test. The values are expressed as means±SD of samples per group. $p<0.05$ was considered significant.

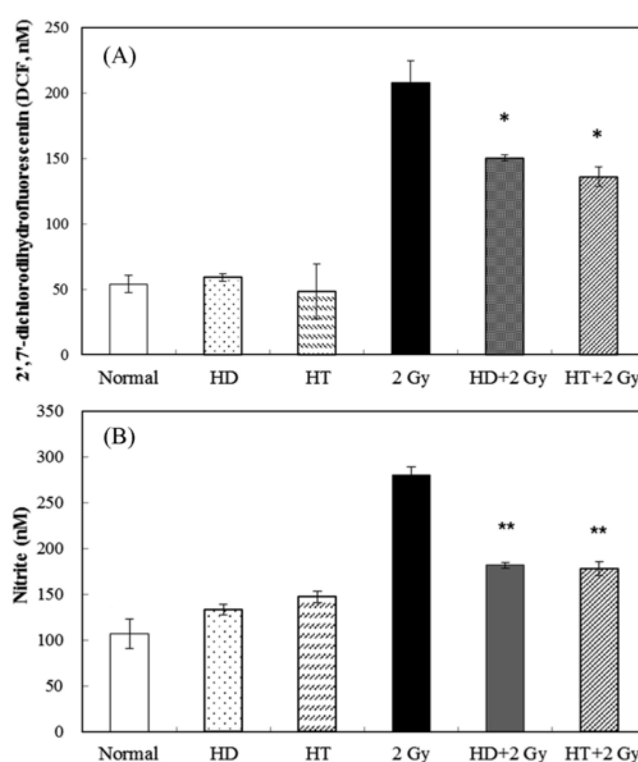

Fig. 2. Effect of hesperetin and hesperidin on radiation induced ROS (A) and NO (B) levels in splenocytes. Splenocytes were treated with hesperetin (500 μM) and hesperidin (500 μM) for 30 min, then irradiated with 2 Gy. Values are expressed as mean±SD of three experiments. *$p<0.05$ and **$p<0.01$ represent significant differences compared with 2 Gy of irradiation group.

Results and Discussion

Cell viability The effect of hesperetin and hesperidin on the splenocytes viability was assessed after irradiation. Cell viability was determined using a WST-1 assay, which is based on the activity of mitochondrial enzyme, succinate dehydrogenase in living cells (16). The cells were treated with different concentrations of hesperetin and hesperidin (20, 100, and 500 μM) to determine the cytotoxic effect on murine splenocytes. Hesperetin and hesperidin did not show cell cytotoxicity compared to the untreated group (Fig. 1A). The murine splenocytes were treated and exposed to two doses of radiation (2 and 4 Gy) and incubated for 24 h. The percentages of proliferated cells were decreased by irradiation in a dose-dependent manner (Fig. 1B). The cell viability of 2 and 4 Gy was about 66.4 and 52.7%, respectively. After pretreatment with hesperetin and hesperidin for 1 h prior to irradiation, cell viability was significantly ($p<0.05$) improved in 500 μM of hesperetin compared with 2 and 4 Gy of the

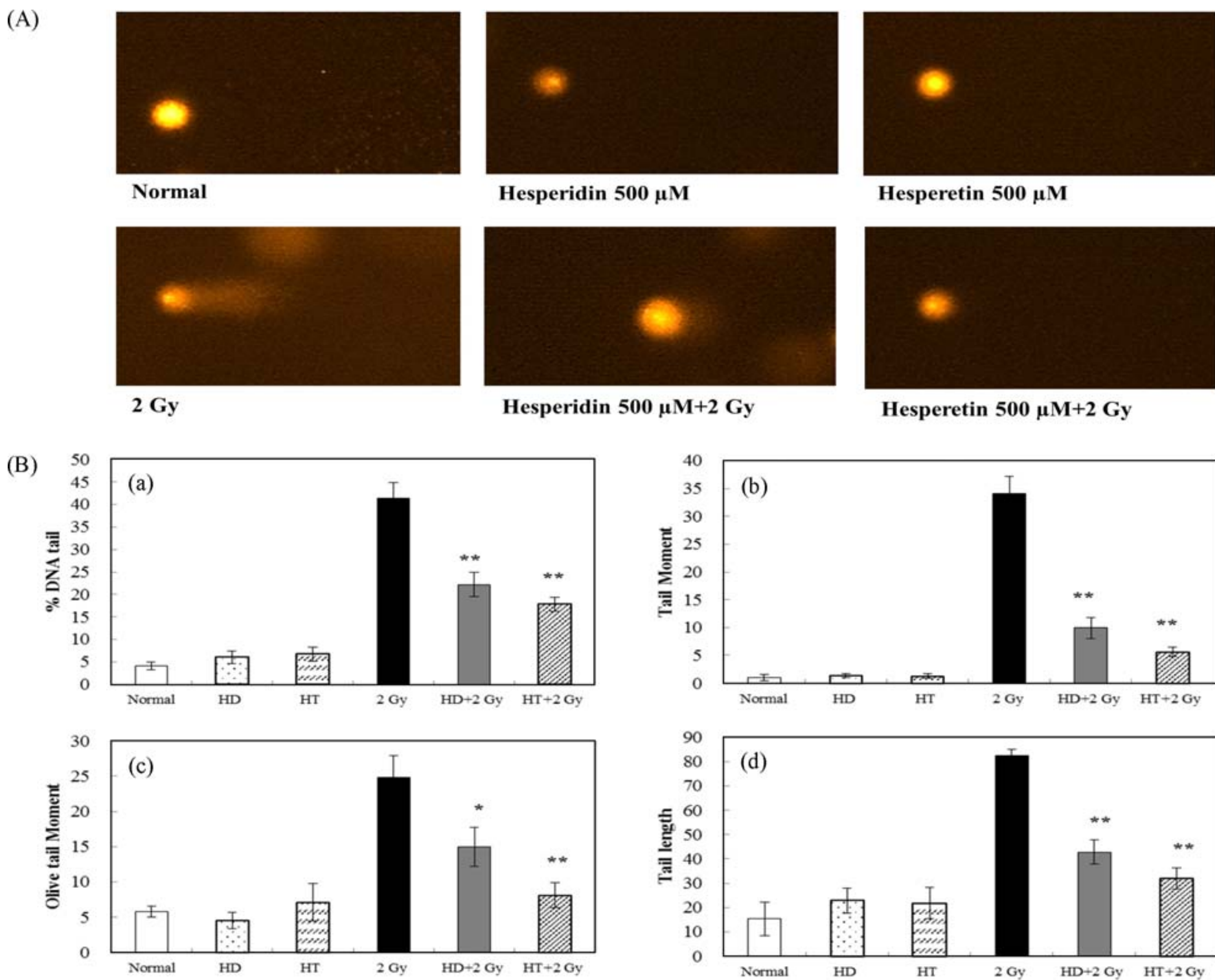

Fig. 3. Effect of hesperetin (500 μM) and hesperidin (500 μM) on DNA damage in splenocytes exposed to 2 Gy of irradiation as analyzed by the comet assay. (A) Comet photos showing the effect of hesperetin on radiation-induced DNA damage in splenocytes. (B) (a) % DNA tail; (b) tail moment; (c) olive tail moment; (d) tail length. It was analysed by using image analysis system. Values are expressed as mean±SD of three experiments. *$p<0.05$ and **$p<0.01$ represent significant differences compared with 2 Gy of irradiation group.

irradiated group (Fig. 1C). The results suggest that hesperetin not only protects radiosensitive cells such as splenocytes against radiation damage.

Intracellular ROS and NO production ROS is considered to play a basic role in irradiation-induced cell death (18). To evaluate the effect of hesperetin and hesperidin on radiation-induced oxidative stress, the levels of ROS production in cells were measured using a DCF probe. The murine splenocytes exposed to 2 Gy of γ-irradiation (208.02±16.57) showed an increase in DCF fluorescence levels when compared with the normal group (54.01±6.67). The DCF levels of hesperetin and hesperidin treatment were 135.80±7.48 and 150.38±2.44, and significantly ($p<0.05$) inhibited the intracellular ROS production in irradiated murine splenocytes (Fig. 2A). Under *in vitro* conditions, hesperetin is reported to act as a free radical scavenger (11). This indicates that hesperetin and hesperidin significantly inhibited radiation-induced intracellular accumulation of ROS by acting as a powerful free radical scavenger. NO is a potent inhibitor of cell proliferation and has been reported to increase radiation-induced damage through the inhibition of the activity of DNA repair enzymes (19). The effects of hesperetin and hesperidin on radiation-induced NO production are shown in Fig. 2B. The measurement of the nitrite concentration is used in NO production. The NO levels of hesperetin and hesperidin treatment were 178.16±13.05 and 181.60±5.26, and significantly ($p<0.05$) reduced of NO production as compared to the irradiated group (280.45± 14.35). Genotoxicity effects of ionizing radiation are also mediated by formation of ROS/NO, which cause DNA strand breaks and mutation (20). These results indicate that hesperetin and hesperidin contributed to the ROS and NO scavenging property against γ-irradiation without cytotoxicity.

Comet assay A comet assay is a very sensitive and highly useful method for detecting DNA damage, including DNA single- and

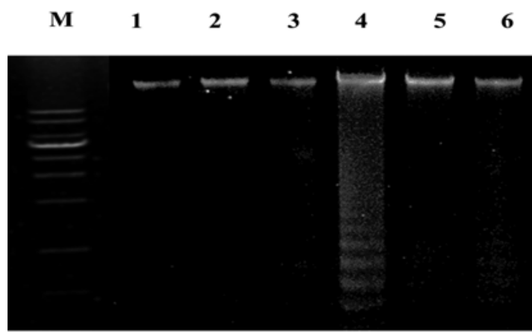

Fig. 4. Effect of hesperetin and hesperidin on radiation-induced DNA damage in the splenocytes using DNA ladder analysis. M, Marker; 1, normal (0 Gy); 2, hesperidin 500 μM; 3, hesperetin 500 μM; 4, 2 Gy alone; 5, hesperidin 500 μM+2 Gy; 6, hesperetin 500 μM+2 Gy.

double strand breaks (21). The DNA damage in γ-irradiation in murine splenocytes is shown in Fig. 3. The extent of DNA damage was calculated based on % DNA tail, tail moment, olive tail moment and tail length in the cells (Fig. 3A and 3B (a)-(d)). There was increase in % DNA tail, tail moment, olive tail moment and tail length in the irradiated murine splenocytes. Hesperetin and hesperidin treatment significantly (p<0.05 or p<0.01) decreased the comet attributes when compared to the irradiated cells. Previous studies have shown that treatment with hesperetin significantly protects from DNA damage by doxorubicin-induced toxicity (22). This indicates that γ-irradiation induces strand breaks and other damage in the cellular DNA, and that hesperetin and hesperidin have the ability to protect murine splenocytes.

DNA ladder assay To confirm further, the effect of hesperetin and hesperidin on radiation-induced DNA damage were detected by a DNA ladder assay. Cells exposed to 2 Gy of γ-irradiation showed a clear visible classic ladder formation. In the normal (0 Gy) group, there was no fragmentation representing the absence of apoptosis. Cells pretreated with hesperetin and hesperidin showed a decrease in ladder formation indicating the inhibition of apoptosis when compared to the irradiated group (Fig. 4).

Cytokine assay Pro-inflammatory cytokines, such as TNF-α and IL-6 have been reported to be significantly increased in the spleen of irradiated mice (23). The levels of pro-inflammatory cytokines in murine splenocytes are shown in Fig. 5. In the normal group, the TNF-α concentration in murine splenocytes was 156.7 pg/mL. The levels of TNF-α after irradiation significantly (p<0.05) increased compared with the normal group, resulting in a concentration of 371.0 pg/mL. However, pretreatment with hesperetin and hesperidin significantly (p<0.05) prevented this high level of TNF-α induced by irradiation, with results of 205.8 and 222.0 pg/mL, respectively (Fig. 5A). The IL-6 concentration of the normal group was 14.2 pg/mL. The levels of IL-6 after irradiation significantly (p<0.05) increased compared

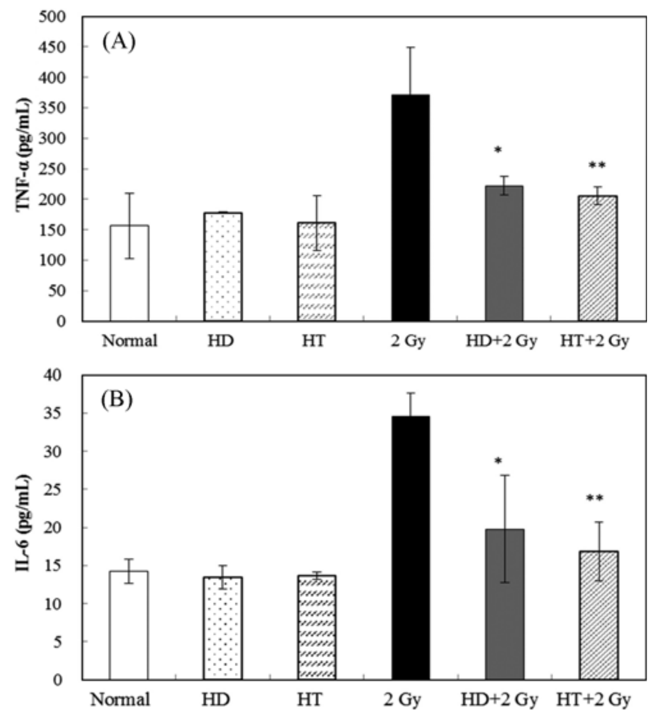

Fig. 5. Effect of hesperetin and hesperidin on radiation induced cytokines TNF-α (A) and IL-6 (B) levels in splenocytes. Splenocytes were treated with hesperetin (500 μM) and hesperidin (500 μM) for 30 min, then irradiated with 2 Gy. Values are expressed as means±SD of three experiments. *p<0.05 and **p<0.01 represent significant differences compared with 2 Gy of irradiation group.

with the normal group, resulting in a concentration of 34.6 pg/mL. However, pretreatment with hesperetin and hesperidin significantly (p<0.05) prevented this high levels of IL-6 induced by irradiation, with results of 16.8 and 19.8 pg/mL, respectively (Fig. 5B). The results of measurements of pro-inflammatory cytokines after irradiation demonstrated an increase in TNF-α and IL-6, and hesperetin pretreatment significantly reduced this elevated parameter. These results may be related to the anti-inflammatory actions of hesperetin.

In summary, our results demonstrate that hesperetin protects the murine splenocytes by scavenging intracellular ROS, limiting DNA damage, enhancing the proliferation of murine splenocytes, and inhibiting pro-inflammatory cytokines. Based on the results of our study, it is our conclusion that hesperetin is a promising candidate radioprotector, particularly for radiotherapy in patients and for space explorers.

Acknowledgments This work was supported by a Nuclear Research Development Program of the National Research Foundation (2012M2A2A6011335) grant funded by the Korea Ministry of Science ICT Future planning.

Disclosure The authors declare no conflict of interest.

References

1. Jagetia GC. Radioprotective potential of plants and herbs against the effects of ionizing radiation. J. Clin. Biochem. Nutr. 40: 74-81 (2007)
2. Hosoda M, Tokonami S, Sorimach A, Monzen S, Osanai M, Yamada M, Kashiwakura I, Akiba S. The time variation of dose rate artificially increased by the Fukushima nuclear crisis. Sci. Rep. 1: 87 (2011)
3. Hosoda M, Tokonami S, Tazoe H, Sorimachi A, Monzen S, Osanai M, Akata N, Kakiuchi H, Omori Y, Ishikawa T, Sahoo SK, Kovacs T, Yamada M, Nakata A, Yoshida M, Yoshino H, Mariya Y, Kashiwakura I. Activity concentrations of environmental samples collected in Fukushima Prefecture immediately after the Fukushima nuclear accident. Sci. Rep. 3: 2283 (2013)
4. Mates JM, Sanchez-Jimenez FM. Role of reactive oxygen species in apoptosis: Implications for cancer therapy. Int. J. Biochem. Cell Biol. 32: 157-170 (2000)
5. Lee W, Ahn G, Lee BJ, Wijesinghe WA, Kim D, Yang H, Kim YM, Park SJ, Jee Y, Jeon YJ. Radio-protective effect of polysaccharides isolated from *Lactobacillus brevis*-fermented *Ecklonia cava*. Int. J. Biol. Macromol. 52: 260-266 (2013)
6. Nair CKK, Parida DK, Nomura T. Radioprotectors in radiotherapy. J. Radiat. Res. 42: 21-37 (2001)
7. Hosseinimehr SJ. Trends in the development of radioprotective agents. Drug Discov. Today 12: 794-805 (2007)
8. Mansour HH, Hafez HF, Fahmy NM, Hanafi N. Protective effect of N-acetylcysteine against radiation induced DNA damage and hepatic toxicity in rats. Biochem. Pharmacol. 75: 773-780 (2008)
9. Garg A, Garg S, Zaneveld LJD, Singla AK. Chemistry and pharmacology of the citrus flavonoid hesperidin. Phytother. Res. 15: 655-669 (2001)
10. Kim JY, Jung KJ, Choi JS, Chong HY. Hesperetin: A potent antioxidant against peroxynitrite. Free Radic. Res. 38: 761-769 (2004)
11. Ameer B, Weintraub RA, Johnson JA, Yost RA, Rouseft RL. Flavanone absorption after naringin, hesperidin and citrus administration. Clin. Pharmacol. Ther. 60: 34-40 (1999)
12. Lee NK, Choi SH, Park SH, Park EK, Kim DH. Antiallergic activity of hesperidin is activated by intestinal microflora. Pharmacology 71: 174-180 (2004)
13. Pradeep K, Park SH, Ko KC. Hesperidin a flavanoglycone protects against gamma-irradiation induced hepatocellular damage and oxidative stress in Sprague-Dawley rats. Eur. J. Pharmacol. 587: 273-280 (2008)
14. Komarova EA, Christov K, Faerman AI, Gudkov AV. Different impact of p53 and p21 on the radiation response of mouse tissues. Oncogene 19: 3791-3798 (2000)
15. Bing SJ, Kim MJ, Park EJ, Ahn G, Kim DS, Ko RK, Lee NH, Shin TK, Park JW, Jee Y. 1,2,3,4,6-Penta-*O*-galloyl-β-D-glucose protects splenocytes against radiation-induced apoptosis in murine splenocytes. Biol. Pharm. Bull. 33: 1122-1127 (2010)
16. Jin YR, Han XH, Zhang YH, Lee JJ, Lim Y, Kim TJ, Yoo HS, Yun YP. Hespretin, a bioflavonoid, inhibits rat aortic vascular smooth muscle cells proliferation by arresting cell cycle. J. Cell. Biochem. 104: 1-14 (2008)
17. Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ. A microplate assay for the detection of oxidative products using 2,7-dichlorefluorescein-diacetate. J. Immunol. Methods 156: 39-45 (1992)
18. Szumiel I. Ionizing radiation induced cell death. Int. J. Radiat. Biol. 66: 329-341 (1994)
19. Smina TP, De S, Devasagayam TPA, Adhikari S, Janardhanan KK. *Ganoderma lucidum* total triterpenes prevent radiation-induced DNA damage and apoptosis in splenic lymphocytes *in vitro*. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 726: 188-194 (2011)
20. Ross GM. Induction of cell death by radiotherapy. Endocr. Relat. Cancer 6: 41-44 (1999)
21. Olive PL. Impact of the comet assay in radiobiology. Mutat. Res. 681: 13-23 (2009)
22. Trivedi PP, Tripathi DN, Jena GB. Hesperetin protects testicular toxicity of doxorubicin in rat: Role of NF-κB, p38 and caspase-3. Food Chem. Toxicol. 49: 838-847 (2011)
23. Zhou D, Yu T, Chen G, Brown SA, Yu Z, Mattson MP, Thompson JS. Effect of NF-kappaB1 (p50) targeted gene disruption on ionizing radiation-induced NF-kappaB activation and TNFalpha, IL-1alpha, IL-1beta and IL-6 mRNA expression *in vivo*. Int. J. Radiat. Biol. 77: 763-772 (2001)