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Radioprotective Effect of Silk Peptide Based on its Immunomodulatory Activities

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Abstract - The objective of this study was to investigate the radioprotective effect of silk peptide (SP) against gamma irradiation-induced damage via its immunomodulatory activity. The proliferation of RAW 264.7 cells, peritoneal macrophage and splenocyte increased upon treatment with SP followed by irradiation in a dose-dependent manner. The TNF-α and IL-6 secretion levels were the highest in irradiated cells treated with 500 μg·ml^{-1} of SP. On the other hand, although the IFN-γ and IL-2 levels were lower in the irradiated cells, the secretion of these cytokines increased when treated with SP. These results demonstrate that SP enhances the immune response by increasing the macrophage activity *in vitro*. The splenocyte's numbers decreased with gamma irradiation, but the administration of SP (200 mg·kg^{-1} B.W.) significantly increased the splenocyte counts. Moreover, the SP treatment significantly increased the IFN-γ and IL-2 secretion in irradiated mice. The mRNA expression results indicate that the SP administration prior to irradiation activated the Th1 cytokine (IFN-γ and IL-2) response as well as the expression of cytokines related to radioprotection (TNF-α, IL-6, and IL-1β). The results also showed that the SP administrated mice survived by 30% more than the PBS feeding group when the mice were irradiated with a lethal dose (6.5 Gy). Thus, the overall results proposed that silk peptide could be a good candidate as radioprotective functional food.

Key words : Radioprotective, Silk peptide, Immune modulation, Cytokine

INTRODUCTION

The silkworm's cocoon is a rich source of protein, as it includes fibroin and sericin. Fibroin is composed of various amino acids such as a 45.9% glycine, 30.3% alanine, 12.1% serine, 5.3% tyrosine, and 1.8% valine. Fibrous proteins such as silks and collagens are depicted by an expensive repetitive primary sequence that leads to significant homogeneity in secondary structures. These types of proteins usually exhibit important mechanical properties in contrast to the catalytic and molecular recognition functions of globular proteins (Altman *et al*. 2003). Silk fibroin has been shown to have physical and chemical properties known for their various physiological effects such as anti-diabetic, hypocholesterolemic, anti-oxidant, immune-regulatory, anti-hangover, anti-HIV, anti-tumor, anti-obesity, wound healing, and hair protecting actions (Nahm *et al*. 1995; Zhang 1995; Gotoh *et al*. 2000; Hong *et al*. 2002; Zhaorigetu *et al*. 2003; Suzuki *et al*. 2004; Roh *et al*. 2006; Hyun *et al*. 2008; Kim *et al*. 2009; Jung *et al*. 2010; Aykac *et al*. 2018). Furthermore, these acid- or enzyme hydrolysate peptides were examined for their biological activities such as effect on the type 2 diabetes blood glucose level (Shin *et al*. 2006) and effect of absorbing alcohol inhibition (Akai 1999). Thus, silk acid- or enzyme hydrolysate peptides have been applied in various fields such as cosmetics,

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medical materials for human health, and food additives (Kato *et al*. 1998). In addition, studies on irradiated silk fibroin or peptides have also been reported (Byun *et al*. 2009; Byun *et al*. 2010).

Exposure to ionizing radiation has caused early or late effects on normal tissues. In addition, depending on the radiation dose, there may be impaired organ function, organ failure, severe tissue reactions, or even death. Radiation-induced tissue or organ damage are mainly produced by bone marrow stem cells that affect hematopoiesis, hence leading to an impaired repair of the lesion because of the reduced rearrangement of functional cells in the irradiated organ (Wondergem and Rosenblatt 2012). Ionizing radiations were triggered by the first step of free radicals' generation, and they are also known as a reactive oxygen species (ROS). These ROS such as OH, H, e^{aq-}, HO_2, and H_3O^+ deplete cellular antioxidant storage and react with cellular macromolecules such as DNA, RNA, proteins, and membrane lipids. They can also cause cell dysfunction or mortality (Dragaric and Dragaric 1971; Pradhan *et al*. 1973; Scholes 1983).

Radioprotectors are defined as agents that are administered before radiation exposure, whereas therapeutic agents are administered after the exposure (Weiss and Landauer 2003). With the increasing use of radiation, the protection of individuals against severe damage by radiation is considered an important issue. Radiation protection is an area of great significance due to its wide applications in planned radiotherapy as well as unplanned radiation exposure (Arora *et al*. 2005; Jagetia *et al*. 2007). Furthermore, it has been demonstrated that various mechanisms such as calcium channel blocking, free radical scavenging, enhancement of DNA repair, inhibition of lipid peroxidation, and stimulation of stem cell proliferation as well as radiation therapy for cancer patients have improved from the use of radioprotectors to protect normal tissue (Hosseinimehr *et al*. 2001). Several studies have also shown that a 5-hydoxytryptamine(5-HT), s-2-(3-amino-propylamino) ethyl phosphorothioic acid (WR-2721) (Miura *et al*. 2000), and prostaglandin were protective actions against irradiation (Walden and Frzaneh 1995). In recent years, radioprotectors have become well-known as antioxidant phytochemicals are present in plants, fruits, herbs, and vegetables (Arora 2005). In addition, synthetic compounds such as antioxidants, cytoprotective agents, immunomodulators, vitamins, and DNA binding molecules, have also been considered as radioprotective potentials (Dorr *et al*. 2001; Bala and Goel 2004; Gonzalez *et al*. 2018). They have also been reported as beneficial for free radical-mediated conditions in humans such as arthritis, atherosclerosis, aging, cancer, Alzheimer's disease, Parkinson's disease, and inflammatory disorders (Arora *et al*. 2006; Jena *et al*. 2010). In particular, the immune-stimulatory activity radiation protector is known for its augment natural killer (NK) cell activity, production of interleukin-1(IL-1), interleukin-2(IL-2), interleukin-6(IL-6), tumor necrosis factor-alpha(TNF-α), and granulocyte macrophage-colony stimulating factor (GM-CSF), as well as increasing the population of CD 3, CD 4, CD 8 cells (Mizuno *et al*. 1994; Ma *et al*. 1995; Gao *et al*. 1996; Agarwal and Singh 1999).

When considering the use of any biomaterial, an immune system reaction is a significant issue. Therefore, the activation of an innate immune cell such as macrophage is a useful determinant of the biocompatibility of biomaterials (Aramwit 2009). Silk peptide has been shown to exhibit various physiological effects such as immunomodulation and antioxidant activities (Park and Hyun 2002; Hwang *et al*. 2019). Moreover, gamma irradiation has been applied to silk fibers to modify structure and mechanical properties and its immunomodulatory efficacy (Byun *et al*. 2010; Yao *et al*. 2011). Nevertheless, the effect of silk peptide on the radioprotection of immunological responses has not been reported yet. In the present study, the wholesome radioprotective effects of silk peptide were investigated *in vivo* and *in vitro* for the whole-body survival, cell proliferation, cytokine secretions, and cytokine mRNA expressions by lethal and sublethal doses of radiation.

MATERIALS AND METHODS

1. Sample preparation

The silk peptide acid hydrolysate (SP) was supplied by World Way Co. Ltd. (Jeonui-myeon, Sejong-si, Republic of Korea) and stored at 4°C. SP was dissolved in a sterilized phosphate buffered saline (PBS) solution.

2. Cell culture

RAW 264.7 cells (macrophage cell line) were purchased from the Korean Cell Line Bank (KCLB). RAW 264.7 cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% fetal bovine serum, 100 unit·ml^{-1} penicillin, and 100 unit·ml^{-1} streptomycin (com-

plete medium) under a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were plated at a density of 5×10^4 cells · well^{-1} in 96 well plates and 1×10^5 cells · well^{-1} in 48 well plates and cultivated to various concentrations of irradiated and non-irradiated doses pretreated with SP (250 and 500 μg · ml^{-1}) at the same above condition. After incubation for 24 h, 96 well plates were measured for cell proliferation. In addition, a 48 well plates cell culture supernatant was harvested for cytokines and nitric oxide production. It was then stored at −70°C. The number of viable cells was determined by trypan blue dye exclusion, and cells were counted with a hemocytometer.

3. Macrophage cell culture from mice

Peritoneal macrophage cells were harvested from un-stimulated 7-week-old BALB/c female mice as described by Kraatz *et al*. (1999). Thioglycollate-elicited peritoneal exudate cells were obtained from mice following the intraperitoneal injection of 1 ml of a thioglycollate broth (2%, Difco Laboratories, Detroit, MI, USA). The lavage liquid from a peritoneal cavity with 10 ml of the Roswell Park Memorial Institute (RPMI) 1640 medium was collected 3 days later. The liquids were washed twice with cold PBS by spin down and resuspended in the complete medium of RPMI-1640. $8 \sim 10 \times 10^6$ cells were seeded on petri dishes (100 × 15 mm), and the macrophages were allowed to adhere for 2 h in a 5% CO_2 humidified atmosphere. The non-adherent cells, which were largely lymphocytes, were then removed with two or three vigorous washes in PBS. The plates were added with PBS containing 0.1 M Trypsin-EDTA (pH 7.0) (Invitrogen Co., CA, USA) to detach the cell. The viability cells were executed by using trypan blue (Invitrogen Co., CA, USA). We used the detached cell as a murine peritoneal macrophage. The counted cells were re-suspended in a complete RPMI-1640 medium and then plated into 24 well (1×10^6 cells · well^{-1}) and 96 well (5×10^5 cells · well^{-1}) culture plates. After incubation for 3 h, the cultured cells were treated with various concentrations (250 and 500 μg · ml^{-1}) in 96 well culture plates, as well as 250 and 500 μg · ml^{-1} concentration of SP in 24 well culture plates. After incubation for 18 h, one of the culture plates (96 well plates and 24 well plates) was irradiated with 20 Gy. After incubation for 24 h, the cell proliferation of 96 well plates were measured by a WST assay. In the case of 24 well plates, the cell culture supernatant was harvested to use for NO and cytokine production and stored at −70°C.

4. Gamma irradiation

The cells and mice were exposed to various irradiation doses (1.5 Gy, 6.5 Gy, 20 Gy, 30 Gy and 150 Gy) from a ^{137}Cs source (Gamma cell 40 Exactor; MDS Nordion International Inc., Ottawa, Ontario, Canada) in the Advanced Radiation Technology Institute of the Korea Atomic Energy Research Institute (Jeoungeup-si, Jeollabuk-do, Republic of Korea). The irradiated cells and mice were incubated at 37°C humidity, 5% CO_2 for indicated periods for further studies. In addition, at least five animals were used per group. The control cells were submitted to the same conditions, but not irradiated.

5. Measurement of NO production using Griess reagent

The concentration of NO in the culture supernatant was determined by measuring nitrite, its oxidation product. In this experiment, the Griess method was employed to detect NO (Di Rosa *et al*. 1990), which is based on the chemical diazotization reaction and uses 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H_3PO_4. $NaNO_2$ was used as the standard curve (0∼100 μM) freshly prepared in deionized water to calculate the nitrite concentration. After 50 μl of the cell culture supernatant was mixed with 50 μl of the Griess reagent, it was incubated at room temperature for 15 min. The absorbance was measured at 595 nm by using a micro plate ELISA reader (Zenyth 3100, Austria).

6. Cytokine productions assay

Supernatants cultured for 24 h were used for the cytokine production test. ELISA kits (BD Biosciencs, San Jose, CA) were used by following the manufacturer's instructions, and interleukin-6 (IL-6), interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) were evaluated with a 450 nm ELISA reader (Zenyth 3100, Austria). The absorbance values were then converted to concentrations (pg · ml^{-1}) of IL-6, IL-2, IFN-γ, and TNF-α using standard curves prepared with serial dilutions of recombinant IL-6, IL-2, IFN-γ, and TNF-α standards.

7. Animals and diets

Female BALB/c (body weight; 19∼21 g), 7-week-old (obtained) mice were obtained from Orient Inc. (Charles River

Technology, Seoul-si, Republic of Korea). For the adaption of vivarium, the mice were housed in a polycarbonate cage in conditions of 22 ± 2°C, a humidity level of 55 ± 10%, and exposure to light and dark in 12 h cycles. In addition, they were also fed a standard animal diet and water for a week. The acclimatized mice were randomly divided into six treatment groups of 10 mice, and from the next day, mice were given an oral administration (200 $mg \cdot kg^{-1}$ Body Wight (B.W.)) of SP for 20 days. After feeding SP, the mice were irradiated to 1.5 Gy. The experimental protocols were approved by the Animal Ethics Committee of the Korea Atomic Energy Research Institute (KAERI-IACUC-2012-011).

8. Splenocyte suspension preparation

To prepare a splenocyte suspension for the *in vitro* study, mice were anesthetized with ether and sacrificed by a cervical decapitation (Kapasi and Singhal 1999). Then the spleen was used to isolate splenocytes and was placed in dishes with a RPMI-1640 medium. The spleen tissues were homogenized by a glass homogenizer (Corning Inc., MA, USA), and the homogenates were then transferred to a sterile universal tube and centrifuged at 600 g at 4°C for 5 min. Thereafter, the supernatant of the homogenate was discarded, and a cell pellet was gently tapped to be resuspended. Two ml of a sterile red blood cell lysis buffer (e-Bioscience Co., San Diego, CA, USA) was added to the cell suspension and incubated at RT for 1 min. Eighteen ml of a RPMI-1640 medium was then added to a cell suspension. The suspension was freed from debris by centrifugation (i.e., 600 g at 4°C for 5 min). This washing step was repeated twice to wash out any residuals from the red blood cell lysis buffer and red blood cells to obtain a splenocyte pellet. Splenocytes were plated in 48 well tissue culture plates at a final concentration of 1×10^6 cells. They were well maintained in a complete RPMI-1640 medium and then cultured at 37°C in 5% CO_2. The cultured supernatant was harvested after 24 h and stored at −70°C for cytokine and nitric oxide production.

9. Splenocyte proliferation and cytokine productions

Spleen tissue was obtained from the irradiation and non-irradiation of pretreatment with SP, respectively. The splenocytes were separated from the same above method described in 2.2.2 splenocyte suspension preparation. Splenocytes were plated in 96 well tissue culture plates at a final concentration of 1×10^6 cells. They were well maintained in a complete RPMI-1640 medium in a 5% CO_2 humidified atmosphere incubator for 24 h. The spleen cell proliferation assay was examined though the same method as the 2.1.2 cell proliferation assay.

Table 1. Oligonucleotides and sequences used in RT-PCR

Oligonucleotide		Sequence
IFN-γ	5′-primer	5′-TACTGCCACGCCACAGTCATTGAA-3′
	3′-primer	5′-GCAGCGACTCCTTTTCCGCTTCCT-3′
IL-12	5′-primer	5′-ACCTCAGTTTGGCCACGGTC-3′
	3′-primer	5′-GTCACGACGCGGGTGGTGAAG-′3
IL-6	5′-primer	5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′
	3′-primer	5′-TCTGACCACAGTGAGGAATGTCCAC-3′
IL-1β	5′-primer	5′-TGAAGGGCTGCTTCCAAACCTTTGACC-3′
	3′-primer	5′-TGTCCATTGAGGTGGAGAGCTTTCAGC
TNF-α	5′-primer	5′-GCGACGTGGAACTGGCAGAAG-3′
	3′-primer	5′-TCCATGCCGTTGGCCAGGAGG-3′
β-actin	5′-primer	5′-TGGAATCCTGTGGCATCCATGAAAC-3′
	3′-primer	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

10. RNA extraction and reverse transcriptase (RT)-PCR

Homogenized spleen samples isolated from BALB/c mice were treated with a Trizol reagent and vortexed after 1/5 volume of chloroform was added. After incubating the mixture at RT for 3 min, the samples were centrifuged at 12,000 g at 4°C for 15 min. The supernatant was transferred to a new 1.5 ml micro centrifuge tube. The RNA from the aqueous phase was precipitated by mixing with the same volume of isopropyl alcohol and centrifuged at 12,000 g at 4°C for 10 min. Precipitated RNA pellets were added to 70% ethyl alcohol at 7,500 g at 4°C for 5 min. The pellet was air dried and dissolved in diethyl pyrocarbonate (DEPC)-treated water. After the RNA clean up using the RNeasy product Mini Kit (Qiagen Inc., Valencia, CA, USA), the RNA was quantitated spectrophotometrically from absorbance at 260 nm. cDNA was synthesized using the cDNA synthesis kit transcription (Roche Applied Science, Penzberg, Germany). The initial denaturation step took place at 94°C for 5 min, the cycling conditions were for 30 cycles at 94°C for 1 min, annealing took place at 55°C for 1 min, and elongation took place at 72°C for 1 min. The last cycle was followed by a 7 min extension step at 72°C. The amplified products (10 μl each) were analyzed by electrophoresis on agarose gels and stained with ethidium bromide (EtBr). The PCR primer sequences are shown in Table 1.

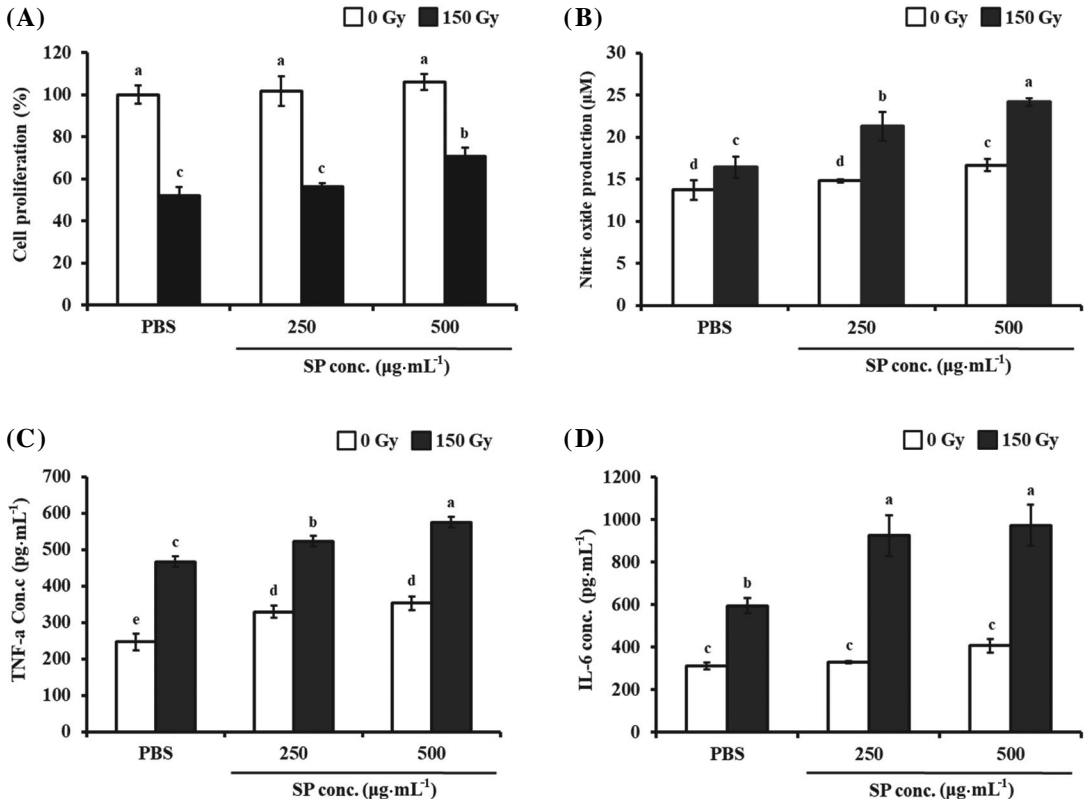

Fig. 1. Effect of silk peptide on RAW 264.7 cell exposed to irradiation. A: RAW 264.7 cell proliferation; B: NO production of RAW 264.7 cell; C: RAW 264.7 cell cytokine (TNF-α) secretion; D: RAW 264.7 cell cytokine (IL-6) secretion. Each experiment was run in triplicate. [a-e]Bars with different letters are significantly different based on Duncan's multiple range test ($p<0.05$).

11. Statistical analysis

One-way analysis of variance was performed using the SPSS software system (2009), and the Duncan's multiple range tests were used to compare the differences among the mean values. The statistical significance of differences between the groups was evaluated for parallel experiments using the Student's t-test. Mean values with standard errors of the mean (SEM) were reported, and the significance was defined at $p<0.05$.

RESULTS AND DISCUSSION

1. Radio protective effect of silk peptide on RAW 264.7 cell

Gamma irradiation exposed (150 Gy) to RAW 264.7 cell pretreated with different concentrations of silk peptide (SP, 250 and 500 $\mu g \cdot ml^{-1}$), as well as cell proliferation, nitric oxide (NO) production, and cytokine (TNF-α and IL-6) secretion were studied to estimate the radio protective effect of silk peptide (Fig. 1). Even though the cell proliferation of control the group that treated PBS reduced significantly by about 50% with gamma irradiation, treatment with 500 $\mu g \cdot ml^{-1}$ of SP resulted in a 1.5-fold increase in the RAW 264.7 cell (Fig. 1A). These findings were in agreement with the previous studies, which reported that silk fibroin hexapeptides had a significant effect on promoting the proliferation of 3T3 preadipocytes *in vitro* (Huang *et al*. 2010) and that the proliferation of hybridoma cells was enhanced on silk sericin (Terada *et al*. 2002).

NO production changes in the SP pretreated RAW 264.7 cell is shown in Fig. 1B. The SP pretreated RAW 264.7 cell showed an increase in NO production, as the concentration was dependent on the gamma irradiated group compared to the PBS treated control. It is assumed that the above result occurred with the increase in NO production at 500 $\mu g \cdot ml^{-1}$ of SP pretreatment in the non-irradiated group. It has been reported that ionizing gamma- or X-ray irradiation can lead to an increased production of NO and an enhanced state of macrophage activation (O'Brien-Ladner *et al*. 1993; Ibuki and Goto 1997; Mckinney *et al*. 1998). NO plays an import-

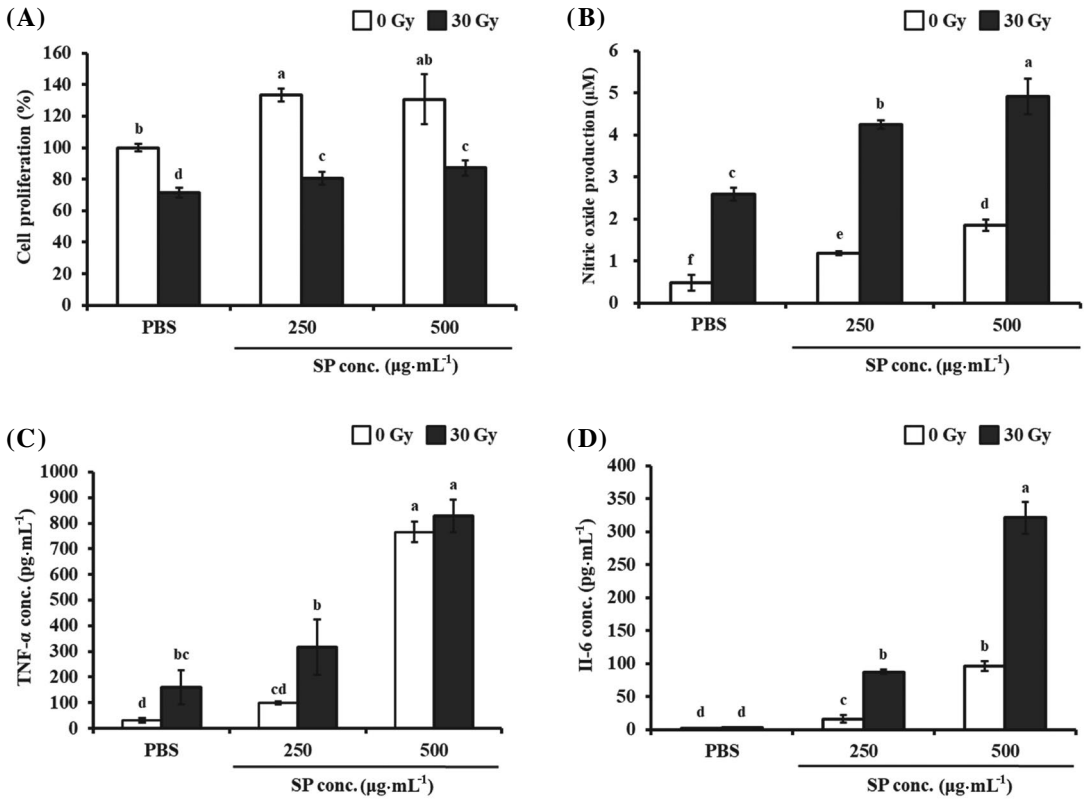

Fig. 2. Effect of silk peptide on murine peritoneal macrophage cell exposed to irradiation. A: Murine peritoneal macrophage cell proliferation; B: NO production of murine peritoneal macrophage cell; C: Murine peritoneal macrophage cell cytokine (TNF-α) secretion; D: Murine peritoneal macrophage cell cytokine (IL-6) secretion. Each experiment was run in triplicate. [a-f]Bars with different letters are significantly different based on Duncan's multiple range test ($p<0.05$).

ant role in the regulating various normal developmental and physiological processes (Gourine *et al*. 2001).

To investigate the role of SP in cytokine secretion induced by gamma irradiation, Fig. 1C and 1D show the levels of cytokines, including TNF-α and IL-6 in non-irradiated and irradiated RAW 264.7 cells. As the non-irradiated RAW 264.7 cells were pretreated with SP secreted high levels of TNF-α, the gamma irradiated group pretreated with SP of 250 and 500 $\mu g \cdot ml^{-1}$ resulted in a significant increase of TNF-α secretion in a concentration dependent manner. The IL-6 secretion level was also elevated by the SP pretreatment compared to the control in the gamma irradiated group. The cytokines' factors modulate the mechanisms of immunologic changes and the immune response method (O' Brien-Ladner *et al*. 1993). TNF-α and IL-6 are potent cytokines produced by many cell types, including macrophages, monocytes, and lymphocytes, in response to inflammation, infections, and injuries. They are also predominantly synthesized and secreted by activated macrophages (Andreakos *et al*. 2002). These results indicated that the NO production and cytokine secretion by SP pretreatment induced RAW 264.7 cell survival from radiation damage.

2. Radio protective effect of silk peptide on murine peritoneal macrophage cell

Cell proliferation, NO production, and cytokine (TNF-α and IL-6) secretion were studied to estimate the radio protective effect of the murine peritoneal macrophage cell pretreated with SP (Fig. 2). Similar to the RAW 264.7 cell result, the cell proliferation of the murine peritoneal macrophage cell was also reduced by 30 Gy of gamma irradiation in control (Fig. 2A). The previous data from Ma *et al*. (2003) have shown that gamma irradiation induces the apoptosis of monocytes and macrophages. However, the SP pretreatment increased the cell proliferation of the murine peritoneal macrophage cell in non-irradiated and gamma irradiated groups.

The NO production of the murine peritoneal macrophage cell increased after the gamma irradiation was in PBS group, and the SP pretreatment also significantly augmented the

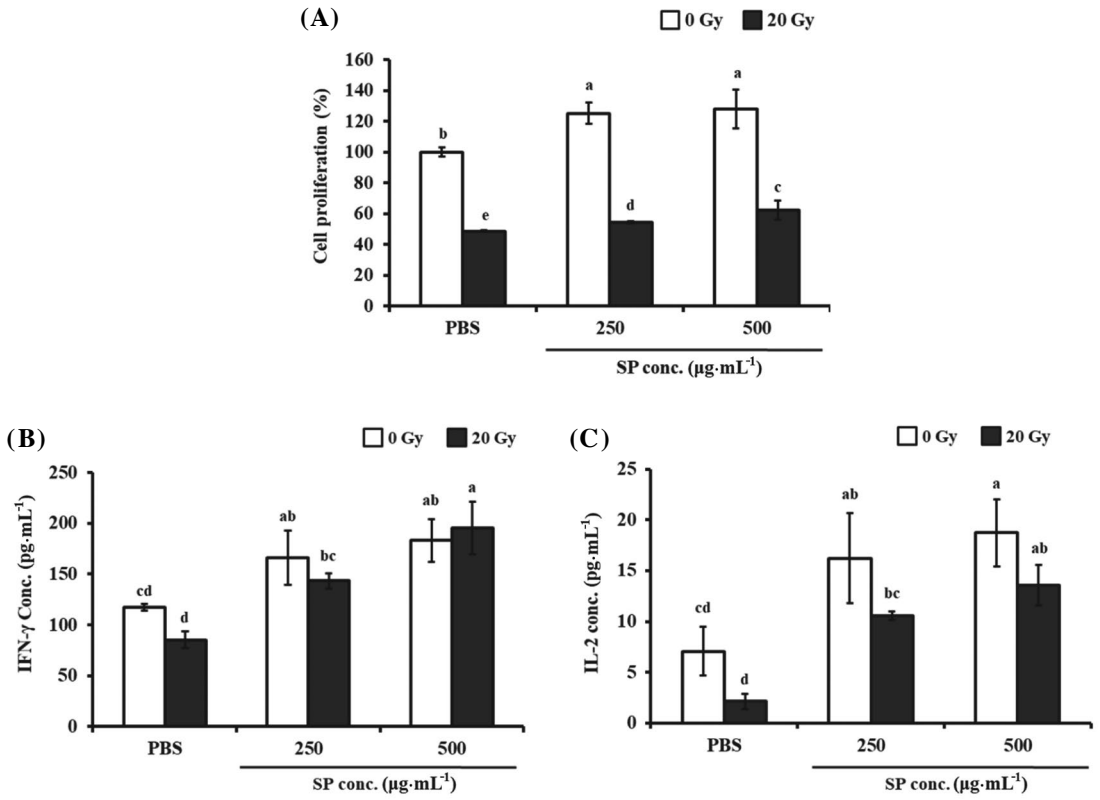

Fig. 3. Effect of silk peptide on cell proliferation and cytokine (IFN-γ and IL-2) secretion in murine splenocyte exposed to irradiation. A: Splenocyte cell proliferation; B: Splenocyte cytokine (IFN-γ) secretion; C: Splenocyte cytokine (IL-2) secretion. Each experiment was run in triplicate. [a-e]Bars with different letters are significantly different based on Duncan's multiple range test ($p < 0.05$).

non-irradiation group (Fig. 2B). The gamma irradiation increased the NO production of SP pretreated murine peritoneal macrophage cell by about 4 times at 250 $\mu g \cdot ml^{-1}$ and 2.5 times at 500 $\mu g \cdot ml^{-1}$ compared to each of the non-irradiated cells. Hildebrandt *et al*. (1998) also reported that gamma irradiation (5∼10 Gy) increased the NO production in macrophages.

The secretion of cytokines (TNF-α and IL-6) in the murine peritoneal macrophage cell is shown in Fig 2C and 2D. Gamma irradiation did not induce IL-6, but TNF-α secretion in the PBS pretreated group. In addition, all of the cytokines secreted more when the SP was pretreated with 250 and 500 $\mu g \cdot ml^{-1}$ in an irradiation or non-irradiation group.

3. Radio protective effect of silk peptide on murine splenocytes

The spleen is known as an important hematopoietic organ in mice (Kojima *et al*., 2000). Cell proliferation and cytokine (IFN-γ and IL-2) secretion without pretreated SP in murine splenocyte are shown in Fig. 3. In order to confirm the radio protective effect of SP, 20 Gy of gamma radiation was used, which was the LD_{50} dose on splenocytes. The pretreatment of SP (250 $\mu g \cdot ml^{-1}$ and 500 $\mu g \cdot ml^{-1}$) significantly increased the cell proliferation of murine splenocytes in the non-irradiation group (Fig. 3A). Even though the cell proliferation of the PBS group on murine splenocyte decreased by half after the gamma irradiation, the SP pretreatment significantly increased the cell proliferation.

The Th1 cells are known to be more radiosensitive than Th2 cells (Galdiero 1994). IL-2 and IFN-γ are mainly secreted by Th1 cells, whereas IL-3, IL-4, IL-5, IL-6, and IL-10 are secreted by Th2 cells (Radford-Smith and Jewell 1996). Fig. 3B and 3C shows the change in the cytokine secretion of the non-irradiation and irradiation groups when the SP is pretreated in the murine splenocyte. Both cytokines secretions were reduced by gamma irradiation in the PBS group, which were similar to a previous study where the ionizing radiation was reduced by cytokine secretion in splenocytes (Oh *et al*. 1997). The non-irradiated SP pretreatment group showed a higher amount of cytokine secretion than the PBS group, and all of the irradiated groups also showed similar results.

4. Radio protective effect of silk peptide on mice exposed to gamma irradiation

After oral administration with two different concentrations of SP (50 and 200 mg·kg^{-1} B.W.) for 20 days, the mice were irradiated with 1.5 Gy. Various immune bio markers (i.e., organ weight, splenocyte cell numbers, splenocyte proliferation, cytokine secretion, and mRNA expression) were studied to confirm the radio protective qualities of SP.

In case of the liver weight of mice, there were no significant differences between administration with PBS and SP or non-irradiated and irradiated (1.5 Gy) mice (Table 2). The spleen weight of non-irradiation mice administrated with PBS and SP also showed the same results as the liver weight result. However, gamma irradiation significantly reduced 40% of the spleen weight of PBS administrated mice. Moreover, SP 200 mg·kg^{-1} B.W. administrated mice showed a significant increase in spleen weight of over 20%, which suggest SP can work as an immunostimulant.

It is well-known that the gamma irradiation induces apoptotic cell death in splenocytes (Chintalwar *et al*. 1999). Our results also showed 1.5 Gy of gamma irradiation reduced the number of splenocyte cells in the PBS administrated group from 11×10^6 cell·ml^{-1} to 4×10^6 cell·ml^{-1} (Fig. 4A). The SP administrated mice exhibited no significant difference in the splenocyte counts compared to the PBS administrated mice in the non-irradiated group. In the case of the irradiation group, even 50 mg·kg^{-1} B.W. of SP administration could not change the splenocyte counts compared to the PBS administrated mice. Two hundred mg·kg^{-1} B.W. of SP ad-

Table 2. Effect of silk peptide on liver and spleen weight in mice splenocytes exposed to irradiation

Irradiation dose	SP concentrations (mg·kg^{-1} B.W.)	Liver	Spleen
		Mean ± SD[a]	Mean ± SD
0 Gy	Control	1.156 ± 0.0598	0.113 ± 0.0064[a]
	50	1.154 ± 0.0301	0.113 ± 0.0134[a]
	200	1.100 ± 0.0703	0.103 ± 0.0121[a]
1.5 Gy	Control	1.132 ± 0.0928	0.069 ± 0.0074[c]
	50	1.117 ± 0.0617	0.068 ± 0.0051[c]
	200	1.099 ± 0.0591	0.083 ± 0.0016[b]

[a-c]Bars with different letters are significantly different based on Duncan's multiple range test ($p<0.05$).

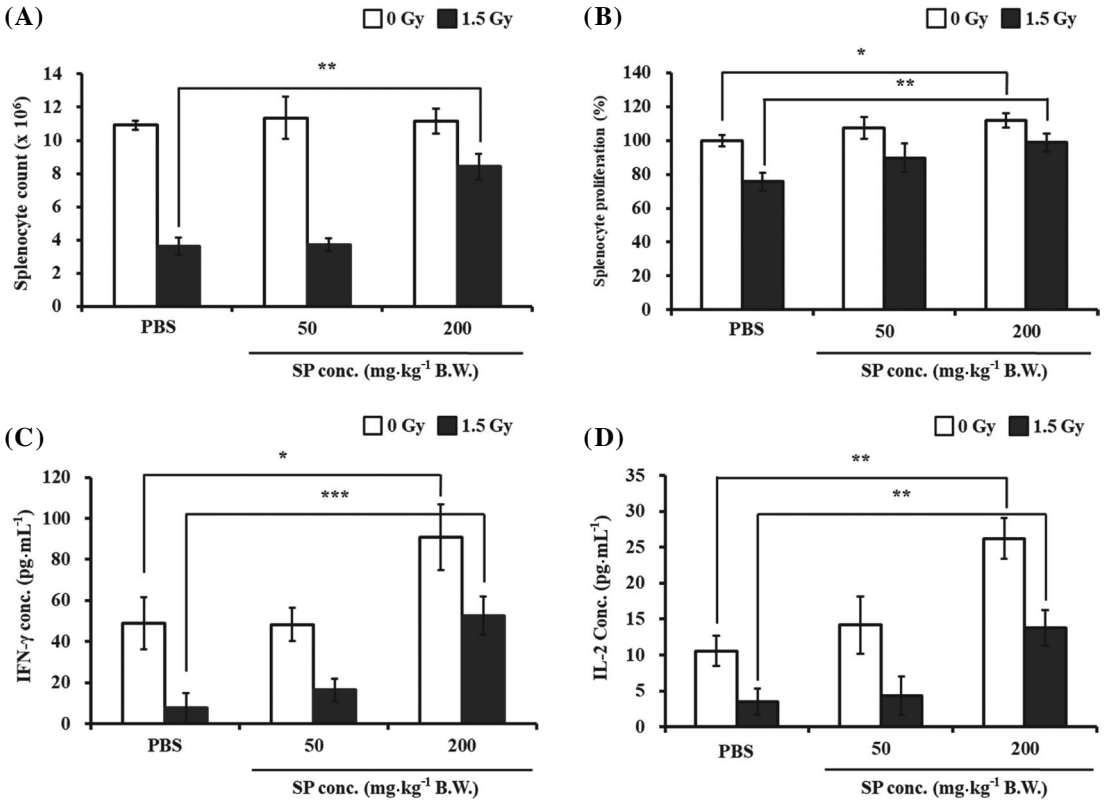

Fig. 4. Effect of silk peptide on total splenocyte count, proliferation, and cytokines (IFN-γ and IL-2) secretion in mice exposed to irradiation. A: Splenocyte total cell count; B: Splenocyte proliferation; C: Splenocyte cytokines (IFN-γ) secretion; D: Splenocyte cytokines (IL-2) secretion. BALB/c mice were administered SP for 20 days before irradiation. *Significantly different from control value based on the student's t-test ($p<0.05$).

ministration significantly increased the splenocyte counts up to 8×10^6 cell · ml^{-1}, which directly referred to the SP protect radiation damage of the spleen.

Though the splenocyte proliferation rate of PBS administrated mice also reduced by about 20% through gamma irradiation, 200 mg · kg^{-1} B.W. of SP administration increased the splenocyte proliferation rate (Fig. 4B). This result indicated that SP can promote the regeneration of splenocytes, which lead to an increasing number of surviving spleen cell numbers.

Fig. 4C and 4D show the levels of splenocyte cytokine secretion (IFN-γ and IL-2) in the SP administrated mice. The levels of both cytokine secretions were significantly reduced by gamma irradiation in the PBS administrated mice. In the non-irradiation group, the SP administration significantly increased the level of both cytokine secretions (IFN-γ and IL-2), especially when 200 mg · kg^{-1} B.W. of SP were administered to mice. This highly elevated level of cytokines was maintained in the irradiation group with 200 mg · kg^{-1} B.W. of the SP administration. The lipid and/or glucose-controlling effects of SP hydrolysates as a result of upregulating lectin and insulin secretion have been reported (Jung *et al*. 2010). The IFN-γ and IL-2 response presumably assists recovery by promoting the restoration of normal functions from irradiation.

The mRNA expression levels of Th1-type (IFN-γ and IL-12) and proinflammatory (IL-1β, IL-6 and TNF-α) cytokines in splenocytes are presented in Fig. 5. The IFN-γ and IL-12 expression levels were strongly upregulated by SP administration when 1.5 Gy of gamma irradiation was induced to mice. The mRNA levels of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) were also elevated by SP administration in a dose-dependent manner. Ishihara *et al*. (1993) reported the effect of irradiation on IL-1β expression in the murine spleen *in vivo*. In general, the radiation-induced production of proinflammatory cytokines (IL-1β and TNF-α) causes radiotherapy-associated disorders in blood (Holler *et al*. 1990). These results indicate that SP administration has an immunostimulatory effect on irradiated mice, which is the consequence of the radioprotective effect.

5. Effect of silk peptide on the survival rate of mice exposed to gamma irradiation

To confirm whether SP could protect mice from radiation-induced lethal damage, the mice were exposed to a lethal dose of 6.5 Gy after the administration of SP at a dose of 200 mg · kg^{-1} B.W. for 20 days (Fig. 6). All of the irradiated animals treated with only PBS died by the 17th day of the 20 days' observation. However, 200 mg · kg^{-1} B.W. of SP administrated mice had a 30% stronger survival rate than the control group. Neta and Oppenheim (1988) reported that the survival of $LD_{95/30}$-irradiated mice from radiation-induced deaths was promoted by IL-1, TNF, and IFN-γ. Song (2003) reported that the radioprotective effects of ginseng treatment could increase the survival of lethally irradiated mice. Therefore, we conclude that SP exerts a protective effect on the immunostimulation factors against radiation damage.

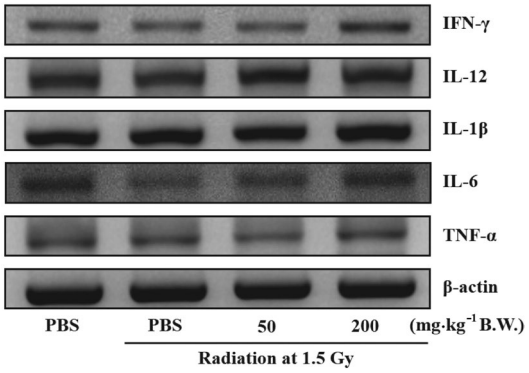

Fig. 5. Effect of silk peptide on cytokine mRNA expression in mice splenocyte exposed to irradiation. BALB/c mice were administered SP for 20 days before irradiation. *Significantly different from control value based on the student's t-test ($p < 0.05$).

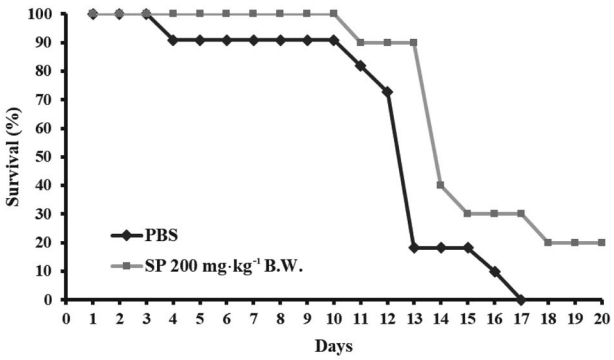

Fig. 6. Effect of Silk peptide on the survival of mice exposed to irradiation (6.5 Gy). BALB/c mice were administered SP for 20 days before irradiation.

CONCLUSION

All of the results of enhanced cell proliferation, immuno-

modulation, stimulation of cytokine expression, and whole-body protection against radiation together likely contributed to the radioprotective efficacy of SP. It might be necessary to further investigate the experimental genotoxiciy, DNA repair, and lipid peroxidation of SP. In conclusion, SP is considered a good candidate of radioprotective functional food through its immunity regulation of the body.

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