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Research article

# Korean Red Ginseng saponin fraction modulates radiation effects on lipopolysaccharide-stimulated nitric oxide production in RAW264.7 macrophage cells

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A R T I C L E I N F O

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A B S T R A C T

*Background:* In previous work, we reported that Korean Red Ginseng saponin fraction (RGSF) showed anti-inflammatory activities *in vitro* and *in vivo*.
*Methods:* The present study investigated the radioprotective properties of RGSF by examining its effects on ionizing radiation (IR)-enhanced and lipopolysaccharide (LPS)-mediated inflammatory responses in murine macrophage cells.
*Results:* RGSF induced strong downregulation of IR-enhanced and LPS-induced proinflammatory responses such as nitric oxide (NO) production (Inhibitory Concentration 50 ($IC_{50}$) = 5.1 ± 0.8 μM) and interleukin-1β levels. RGSF was found to exert its radioprotective effects by inhibition of a signaling cascade that activated checkpoint kinase 2–nuclear factor-κB. In addition, RGSF strongly inhibited IR-enhanced LPS-induced expression of hemoxyganase-1, implying that the latter may be a potential target of RGSF.
*Conclusion:* Taken together, our data suggest that RGSF can be considered and developed for use as an effective radioprotective agent with minimal adverse effects.
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## 1. Introduction

Macrophages, a key player in inflammatory responses, are radioresistant and their functions are not altered by a single radiation treatment [1,2]. In fact, some studies have reported that radiation can boost macrophage stimulation. Gallin et al have reported that J774.1 macrophage cells show enzymatic and morphological changes, and cell activation by 20 Gy ionizing radiation [3]. Indeed, Lambert and Paulnock have reported that radiation increased sensitivity to lipopolysaccharide (LPS) and antigen expression of major histocompatibility complex class I in peritoneal macrophages and RAW264.7 cells, and these changes primed the cell to induce a tumoricidal effect [4]. In addition, production of some cytokines and their mRNA expression have been reported after irradiation in mouse spleen macrophages and human alveolar macrophages [5–7].

Ionizing radiation (IR) induces reactive oxygen species production and DNA damage in cells [8]. As a result, many signaling pathways are activated, such as p53 and ataxia telangiectasia mutated (ATM) kinase, for restoration of radiation-induced DNA damage [9]. Some previous studies have reported that radiation can potentiate LPS-induced production of nitric oxide (NO) through a DNA damage effect, but not reactive oxygen species production. Yoo et al [10] have reported that γ-irradiated (5–40 Gy) murine embryonic liver cells show enhanced production of NO; a widely researched gaseous free

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**Table 1**
Primers of the Investigated Genes in RT-PCR Analysis

| Gene | | Primer sequences |
|---|---|---|
| IL-1β | F | 5′- CAG GAT GAG GAC ATG AGC ACC-3′ |
| | R | 5′- CTC TGC AGA CTC AAA CTC CAC-3′ |
| GAPDH | F | 5′- GTG GGC CGC CCT AGG CAC CAG -3′ |
| | R | 5′- GGA GGA AGA GGA TGC GGC AGT -3′ |

F, forward; GAPDH, glyceraldehydes-3-phosphate de hydrogenase; IL, interleukin; R, reverse; RT-PCR, reverse transcription-polymerase chain reaction.

radical that shows tumoricidal activity, due to hydrogen peroxide formation. In addition, Yuko et al have reported that γ-irradiated RAW264.7 cells show enhanced production of NO and DNA damage via the nuclear factor (NF)-κB pathway [11]. In this regard, we thought that this *in vitro* system, γ-irradiated enhancement of NO production, could be a good model for study of the functional role of new candidates for radioprotective properties.

Recently, interest in the use of natural products for development of potential candidate drugs for protection against radiation exposure has been growing. Phytotherapeutic agents with the capacity to modulate the radiation effect and reduce the subsequent tissue damage are required, while minimizing side effects. In our previous work, we demonstrated the anti-inflammatory effects of the 20(S)-protopanaxdiol (PPD)-rich fraction of ginseng in LPS-induced RAW264.7 cells [12]. However, little is known about the radioprotective properties of the PPD-rich fraction of ginseng. Therefore, we examined the radioprotective properties and molecular mechanisms of PPD-rich red ginseng saponin fraction (RGSF) on the release of proinflammatory indicators in γ-irradiation enhanced LPS-stimulated RAW264.7 murine macrophage cells.

## 2. Materials and methods

### 2.1. Materials

Korean Red Ginseng was kindly provided by the Research Institute of Technology, Korea Ginseng Corporation. Reverse transcriptase (RT) and polymerase chain reaction (PCR) premixes were purchased from Bioneer (Daejon, Korea). LPS was purchased from Sigma (St Louis, MO, USA). All other chemicals and materials were purchased from Sigma—Aldrich, unless indicated.

### 2.2. RGSF extraction and preparation

RGSF extraction was performed as described previously [12,13]. Korean red ginseng was extracted with ethanol and the extract was

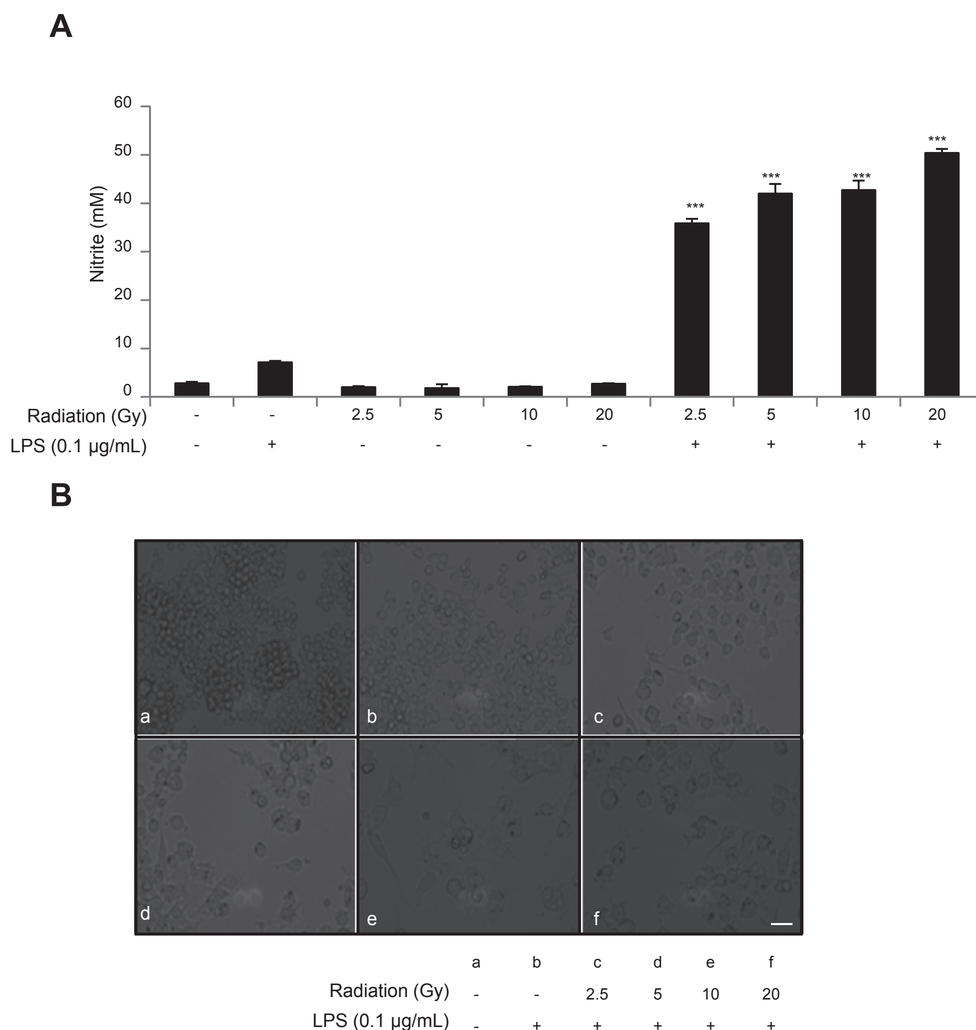


**Fig. 1.** IR enhances LPS-induced production of NO in RAW264.7 cells. RAW264.7 cells ($5 \times 10^4$ cells/mL) were irradiated using the indicated doses using a blood γ irradiator and incubated at 37°C for 24 h. Irradiated cells were stimulated with 0.1 μg/mL LPS for 24 h. (A) The culture supernatant was used for $NO_2^-$ determination using Griess reagent. (B) Corresponding microscopic images (200×) of RAW264.7 cells showed that irradiated cells were widened and dendrite formation was enhanced. Scale bar = 50 μm. IR, ionizing radiation; LPS, lipopolysaccharide; NO, nitric oxide. ***$p < 0.001$ versus LPS.

air dried at 60°C for 2 d. The powder was then subjected to aqueous extraction three times at 95–100°C. The resultant water extracts were ultrafiltered with a pore size of 100,000 μm. Finally, the filtrate was recovered as RGSF for further identification of major chemical components (PPD saponins) by high-performance liquid chromatography profile analysis.

### 2.3. Cell culture

RAW264.7 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured at 37°C in 5% $CO_2$/95% air in Dulbecco's modified Eagle's medium (Welgene, Daegu, Korea) containing 10% fetal bovine serum, and a penicillin (100 U/mL)/streptomycin (100 μg/mL) solution.

### 2.4. Cell irradiation

Cells were irradiated with γ rays from a Biobeam 8000 ($^{137}$Cs source) (Gamma-Service Medical GmbH, Leipzig, Germany) at a dose rate of 2.5 Gy/min at room temperature. Following irradiation, cells were incubated at 37°C for the indicated times.

### 2.5. NO assay

RAW264.7 cells ($5 \times 10^4$ cells/mL) were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and irradiated (10 Gy) using a blood γ irradiator and incubated at 37°C for 24 h. Cells were then washed twice with phosphate-buffered saline (PBS). Cells were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and stimulated by LPS (0.1 μg/mL) for 24 h. The culture supernatant was used for nitric dioxide ($NO_2^-$) determination using Griess reagent. Equal volumes of culture supernatant and Griess reagent were mixed and the absorbance was determined at 570 nm using a PARADIGM Detection Platform ELISA plate reader (Beckman Coulter, Fullerton, CA, USA).

### 2.6. Cell viability test

Cell viability test was performed based on the reduction of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent into an insoluble, dark purple formazan product in viable cells in order to evaluate the cytotoxic effect of RGSF. RAW264.7 cells ($1 \times 10^5$ cells/mL) were incubated with RGSF (0, 2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 24 h. Then, 50 μL of 2 mg/mL MTT reagent was added to the culture plates and further incubated at 37 °C for 2 h and the absorbance was determined at 570 nm using a PARADIGM Detection Platform ELISA plate reader.

### 2.7. Total RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from RAW264.7 cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The extracted total RNA was then used for

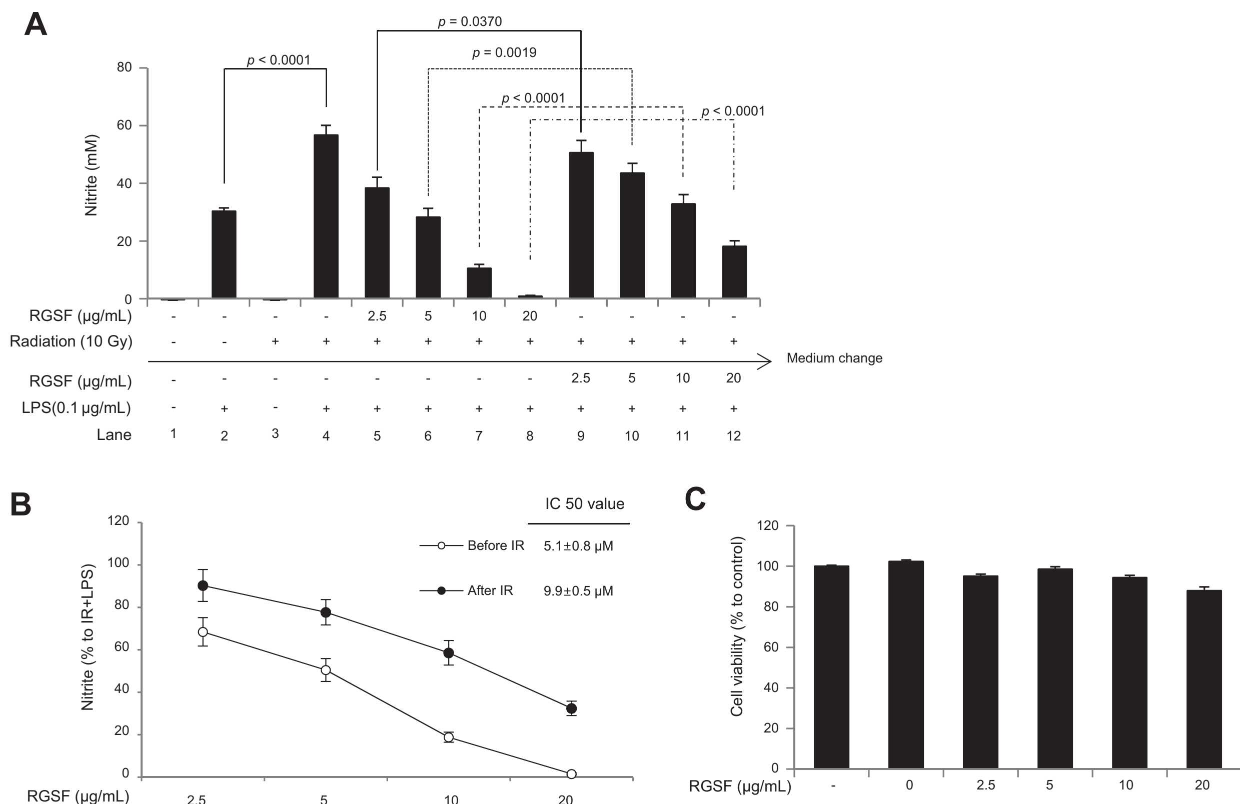


**Fig. 2.** Effect of RGSF on cell viability and production of NO in IR-enhanced LPS-stimulated RAW264.7 macrophages. (A) RAW264.7 cells ($5 \times 10^4$ cells/mL) were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and irradiated (10 Gy) using a blood γ irradiator and incubated at 37°C for 24 h. Cells were washed twice with phosphate-buffered saline. Cells were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and stimulated with LPS (0.1 μg/mL) for 24 h. The culture supernatant was used for $NO_2^-$ determination using Griess reagent. Each bar graph represents mean ± standard error of the mean of at least four independent experiments. *$p < 0.05$, **$p < 0.01$ versus the IR- and LPS-treated groups. (B) Percentage inhibition of RGSF on NO production was analyzed before and after treatment with RGSF. (C) RAW264.7 cells ($1 \times 10^5$ cells/mL) were incubated with RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 24 hours. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed for evaluation of the cytotoxic effect of RGSF. IR, ionizing radiation; LPS, lipopolysaccharide; RGSF, red ginseng saponin fraction.

semiquantitative RT-PCR using RT premix (Bioneer). Briefly, 2 μg of total RNA was incubated with oligo-$dT_{18}$ at 70°C for 5 min and cooled on ice for 3 min, followed by incubation of the reaction mixture containing RT premix for 90 min at 42.5°C, with final inactivation of RT at 95°C for 5 min. The PCR was continued using a PCR premix (Bioneer) with target-gene-specific primers (Table 1).

### 2.8. Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 cells ($5 \times 10^4$ cells/mL) were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and irradiated (10 Gy) using a blood γ irradiator and incubated at 37 °C for 24 h. Cells were then washed twice with PBS. Cells were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and stimulated with LPS (0.1 μg/mL) for 24 h. Cytokine levels in the culture supernatant were evaluated using an IL-1β ELISA kit following the manufacturer's protocol (BD, Franklin Lakes, CA, USA).

### 2.9. Luciferase reporter gene activity assay

RAW264.7 cells ($2 \times 10^6$ cells/mL) were transfected with 10 μg plasmid containing NF-κB-Luc, AP-1-Luc, and TK-renilla-Luc using electrophoresis according to the manufacturer's instructions (Neon Transfection System; Invitrogen, Carlsbad, CA, USA). The cells were used for experiments 24 h after transfection. Luciferase assay was performed using the Luciferase Assay System (Promega, Madison, WI, USA) as reported previously [14].

### 2.10. Immunoblotting

After the indicated treatment in RAW264.7 cells was terminated, total proteins were prepared using Pro-prep lysis buffer (iNtRON, Seoul, Korea) according to the manufacturer's instructions. Concentrations of the extracted proteins were determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA); 50 μg proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The blots were blocked with Tris-buffered saline and Tween 20 containing 5% skimmed milk (Blotto; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and probed with primary antibody diluted in 5% bovine serum albumin (Santa Cruz Biotechnology). The immunoblots were incubated with horseradish peroxidase secondary antibody, and antibody binding was visualized using enhanced chemiluminescence (ECL Plus Western Blotting Detection Reagent GE Healthcare, Little Chalfont, UK).

### 2.11. Statistical analysis

Data were represented as the mean ± standard error of the mean (SEM) of at least three independent experiments, performed in triplicate. Student's *t* test was carried out to analyze the statistical significance between the groups using SPSS version 18.0 (SPSS, Chicago, IL, USA). A $p$ value $< 0.05$ was considered statistically significant.

## 3. Results and discussion

### 3.1. Effect of IR on LPS-stimulated production of NO in RAW264.7 murine macrophage cells

To determine whether IR could enhance the NO-producing capability of signals of LPS, RAW264.7 cells were first irradiated with different doses of radiation (0 Gy, 2.5 Gy, 5 Gy, 10 Gy, and 20 Gy; 2.5 Gy/min) and then left without further treatment or exposed to LPS (0.1 μg/mL) for 24 h postirradiation. As shown in Fig. 1A, increased NO production was observed in irradiated cells in response to LPS at doses as low as 2.5 Gy. Meanwhile, treatment with radiation alone did not induce measurable NO production (data not shown). The maximal effect of radiation was observed at 20 Gy. This LPS signal-boosting effect of γ-irradiation on NO production was only manifested if the radiation was applied 24 h prior to addition of LPS, which was consistent with the results reported by Mckinney et al [15]. Microscopic examination (Fig. 1B) also showed that irradiated macrophages were widened and dendrite formation was enhanced by IR prior to LPS stimulation.

### 3.2. RGSF strongly downregulates IR-enhanced NO production

To examine the question of whether RGSF could modulate the radiation effect on LPS-induced production of NO in RAW264.7 cells, cells were preincubated with RGSF for 10 min prior to IR (10 Gy) treatment and further incubated for 24 h. On the following day, RGSF was washed out with PBS twice before LPS stimulation.

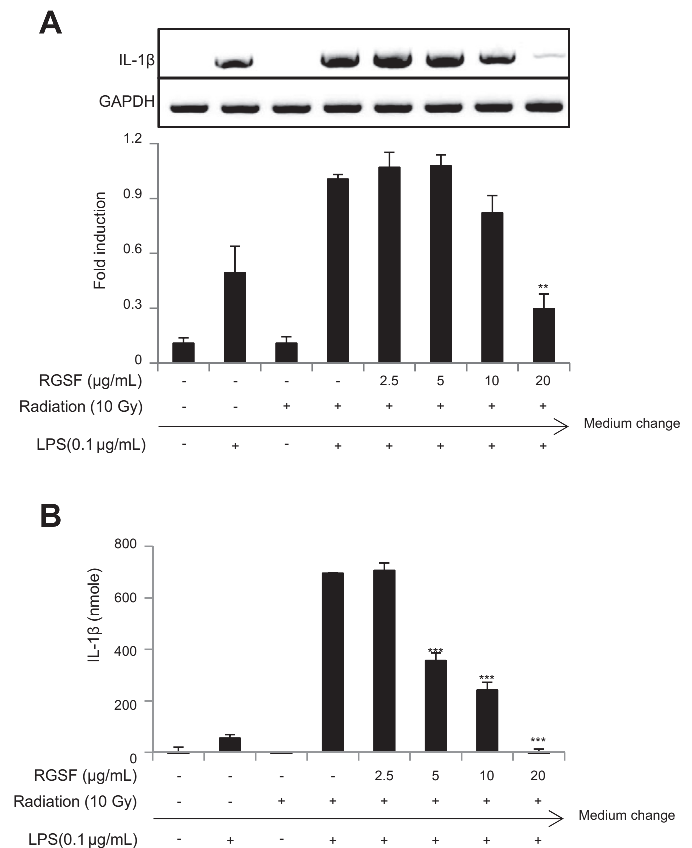


**Fig. 3.** Effect of RGSF on IL-1β mRNA and protein levels on IR- and/or LPS-stimulated RAW264.7 cells. RAW264.7 cells were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and irradiated (10 Gy) using a blood γ irradiator and incubated at 37°C for 24 h. Cells were washed twice with phosphate-buffered saline and stimulated with LPS (0.1 μg/mL) for 24 h. mRNA and protein levels for IL-1β were evaluated by (A) semiquantitative polymerase chain reaction and (B) enzyme-linked immunosorbent assay, respectively. (A) The results shown are representative of three independent experiments and a bar graph. The graph represents the mean ± standard error of the mean of at least three independent experiments. (B) Data are presented as the mean ± SEM of at least three independent experiments. GAPDH, glyceraldehyds-3-phosphate dehydrogenase; IL, interleukin; IR, ionizing radiation; LPS, lipopolysaccharide; RGSF, red ginseng saponin fraction. *$p < 0.05$, **$p < 0.01$ compared to the IR + LPS-treated groups.

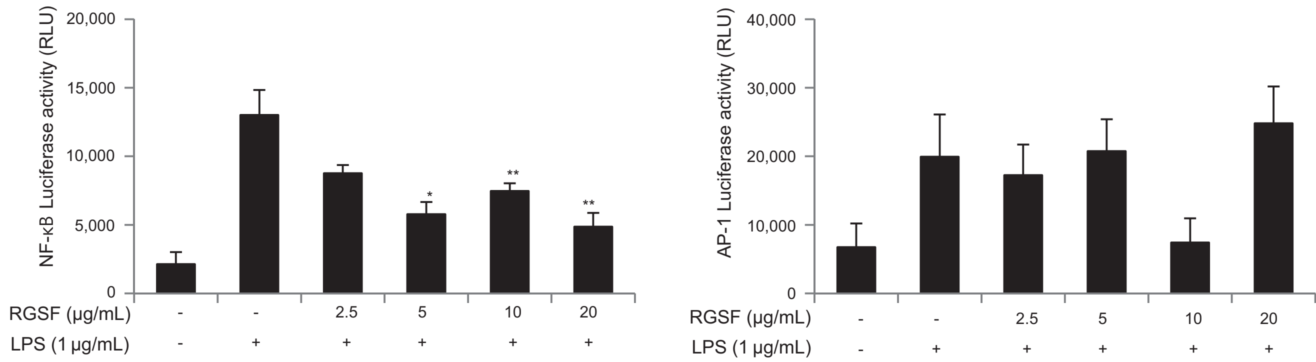


**Fig. 4.** Effect of RGSF on proinflammatory transcriptional factor activation. RAW264.7 cells were cotransfected with the plasmid constructs nuclear factor-κB, activator protein-1, and TK-Renilla using an electroporator. Cells were treated with the indicated concentrations of RGSF in the absence or presence of LPS (1 μg/mL) for 6 hours. Cells were then lysed and luciferase promoter activity was determined using a luminometer. Each bar graph represents mean ± standard error of the mean of at least four independent experiments. LPS, lipopolysaccharide; RGSF, red ginseng saponin fraction. $^{*}p < 0.05$ versus LPS.

Therefore, we investigated the question of whether RGSF can differentially affect inflammatory response in LPS-alone- and IR + LPS-stimulated RAW264.7 cells. As shown in Fig. 2A, pre-treatment with irradiation (10 Gy) resulted in a greater than twofold increase in LPS-induced production of NO, compared with activation of RAW264.7 cells with LPS alone. This IR (10 Gy)-enhanced LPS-induced production of NO showed a significant and concentration-dependent reduction by pretreatment with RGSF prior to radiation treatment. However, treatment with RGSF after radiation resulted in a less-effective reduction of NO production, compared to RGSF pretreatment before radiation in LPS-stimulated RAW264.7 cells. The inhibitory profiles of RGSF on NO production before and after treatment with RGSF against radiation insult were comparable with different potency (Fig. 2A). The Inhibitory Concentration 50 ($IC_{50}$) value pre- and post-treatment with RGSF on IR-enhanced LPS-induced production of NO was 5.1 ± 0.8 μM and 9. 9 ± 0.5 μM, respectively (Fig. 2B). These results strongly suggest that pretreatment with RGSF protects macrophages from radiation effects that boost NO production signaling. In addition, these observed inhibitory effects were not due to RGSF cytotoxicity at all concentrations used (Fig. 2C).

### 3.3. *RGSF attenuates IR-induced expression of IL-1β mRNA and protein secretion*

Excessive production of NO is closely related to abundant induction of various inflammatory cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α. Among them, IL-1β, a proinflammatory cytokine, has already been shown to contribute to radiation injury [16] and inhibition of IR-induced or IR-enhanced IL-1β levels is considered essential for protection from IR-induced damage. Therefore, we investigated the effect of RGSF on IR-enhanced LPS-induced expression of IL-1β mRNA and protein secretion levels using semiquantitative RT-PCR and ELISA,

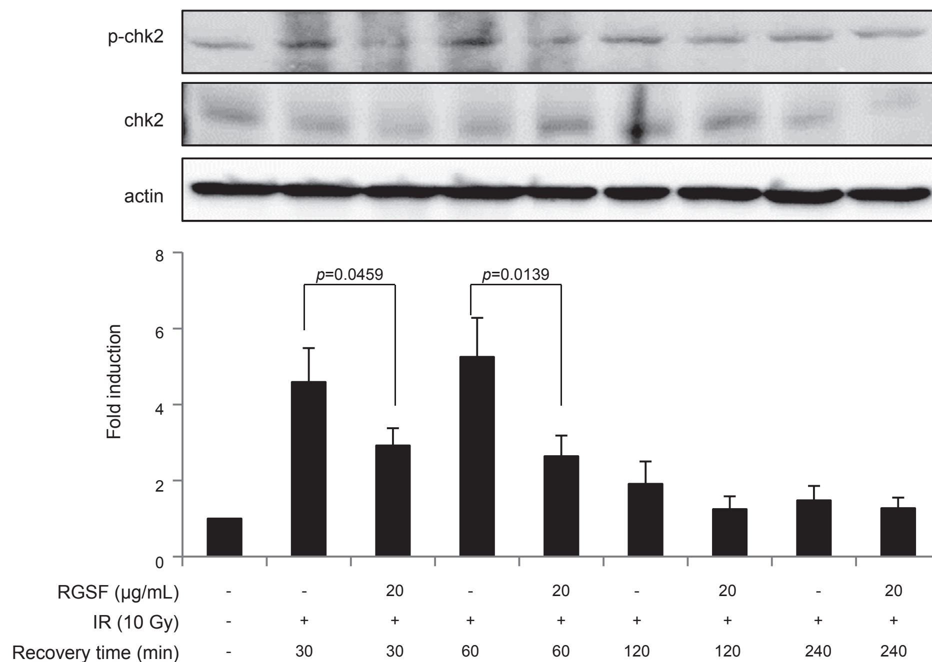


**Fig. 5.** Effect of RGSF on DNA damage related gene activation. RAW264.7 cells ($1 \times 10^6$ cells/mL) were incubated with or without RGSF (20 μg/mL) for 10 min and irradiated (10 Gy) using a blood gamma irradiator and incubated at 37°C for 30 min. Cells were then lysed and immunoblotting was performed. RGSF, red ginseng saponin fraction.

respectively. As shown in Fig. 3A and B, radiation insult resulted in enhanced LPS-induced expression of IL-1β at the levels of mRNA and protein. However, levels of other cytokines, such as tumor necrosis factor-α and cyclooxygenase-2, were not significantly changed by IR, compared to the LPS-only treated group (data not shown). Pretreatment with RGSF resulted in strongly attenuated IR-enhanced LPS-induced IL-1β levels in a concentration-dependent manner. As a result, we confirmed that RGSF strongly attenuated IR-induced excessive inflammatory injury-related markers (NO and IL-1β).

### 3.4. RGSF inhibits NF-κB transcriptional activity

The fact that inflammatory mediator production (i.e., NO and cytokines) is mainly modulated at the transcriptional level, such as NF-κB and activating protein (AP)-1 transcription factors, is well established [17–20]. Indeed, Yuko et al have reported that NF-κB is a key regulator of radiation-enhanced LPS-induced production of NO [11]. Therefore, we explored the question of whether RGSF could modulate agonist-induced NF-κB transcriptional activity of AP-1. RAW264.7 cells were transiently transfected with NF-κB-Luc/TK-renilla plasmids using electrophoresis. In the following days, the cells were stimulated with LPS (1 μg/mL) for 7 h with or without RGSF pretreatment, and NF-κB transcriptional activity was determined. As shown in Fig. 4, RGSF induced notable repression of NF-κB activation in a concentration-dependent manner. However, RGSF had no effect on activity of AP-1, another important redox-sensitive transcriptional factor. This result suggests that RGSF protects cells from radiation-induced DNA damage via inhibitory regulation of NF-κB activity.

### 3.5. RGSF inhibits IR-induced Chk2 activity

Chk2 is another widely studied radiation-induced, DNA-damage-related gene that is an effector of ATM, a regulator of DNA damage checkpoints in mammalian cells [21] and an upstream molecule of radiation-induced NF-κB activation pathways [22]. Therefore, we examined the effect of RGSF on IR-induced activity of chk2. As shown in Fig. 5, pretreatment with RGSF resulted in attenuation of IR-induced phosphorylation of chk2. This suggests that chk2 is an upstream target of RGSF in IR-induced DNA damage.

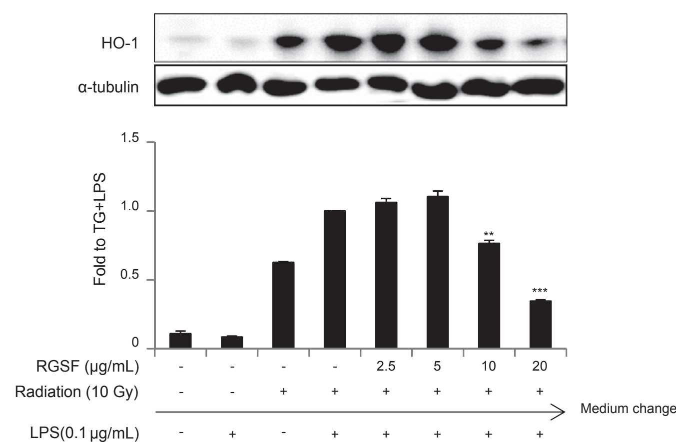


**Fig. 6.** Effect of RGSF in ionizing-radiation-enhanced HO-1 expression against LPS stimulation in RAW264.7 cells. RAW264.7 cells were incubated with or without RGSF (2.5 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL) for 10 min and irradiated (10 Gy) using a blood γ irradiator and incubated at 37°C for 24 h. Cells were then washed twice with phosphate-buffered saline and stimulated with LPS (0.1 μg/mL) for 24 h. HO-1 expression levels were identified by immunoblotting. Data are representative of three independent experiments. HO, hemoxygenase; LPS, lipopolysaccharide; RGSF, red ginseng saponin fraction.

### 3.6. RGSF downregulates IR-enhanced HO-1 expression levels

HO is an enzyme that catalyzes the degradation of heme into iron, biliverdin, and carbon monoxide [23]. The HO family consists of three subtypes, HO-1, HO-2, and HO-3. Among them, HO-1 is a redox-sensitive and ubiquitous inducible stress protein [24,25], which plays a protective role against various cellular stress conditions [26–28]. Recently, growing evidence has indicated that IR can enhance HO-1 expression [29,30]. This is regarded as a biomarker of radiation-induced damage. To elucidate the mechanism of the inhibitory effects of RGSF on radiation-enhanced LPS-induced production of NO in RAW264.7 cells, we examined the question of whether RGSF could affect HO-1 protein expression levels. As shown in Fig. 6, LPS did not affect HO-1 expression levels; however, radiation treatment (10 Gy) resulted in markedly increased expression levels of HO-1 protein. This result is in accordance with those of other studies [27,29]. Of particular interest, pretreatment of IR prior to LPS resulted in clearly enhanced expression of HO-1, more than that of macrophage cells treated with radiation only. This result is exactly in line with NO production trends. In addition, RGSF induced a concentration-dependent decrease in levels of IR-enhanced LPS-induced expression of HO-1. These findings confirmed that RGSF downregulates radiation-induced tissue damage in RAW264.7 cells.

Collectively, these data showed that PPD-rich RGSF can strongly attenuate the augmentation of IR-enhanced LPS-induced production of NO via inhibition of the chk2, NF-κB, and HO-1 signaling pathways. To the best of our knowledge, this is the first report on the radioprotective activity of RGSF using an *in vitro* macrophage system and it offers new insights into the radioprotective characteristics of RGSF. However, data pertaining to the associated receptors and exact intracellular mechanisms of RGSF during radiation response remain elusive. Thus, conduct of further studies is needed in order to clarify the exact molecular mechanisms underlying RGSF-induced downregulation of HO-1.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgments

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