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β-sitosterol Decreases Irradiation-Induced Thymocyte Early Damage by Regulation of the Intracellular Redox Balance and Maintenance of Mitochondrial Membrane Stability

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Abstract Both radiation injury and oxidation toxicity occur when cells are exposed to ion irradiation (IR), ultimately leading to apoptosis. This study was designed to determine the effect of β-sitosterol (BSS) on early cellular damage in irradiated thymocytes and a possible mechanism of effect on irradiation-mediated activation of the apoptotic pathways. Thymocytes were irradiated (6 Gy) with or without BSS. Cell apoptosis and apoptosis-related proteins were evaluated. BSS decreased irradiation-induced cell death and nuclear DNA strand break while attenuating intracellular reactive oxygen species (ROS) and increasing the activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). BSS decreased the release of cytochrome *c* from mitochondria to the cytosol and the mitochondrio-nuclear translocation of apoptosis-inducing factor (AIF). Furthermore, BSS partially inhibited the radiation-induced increase of cleaved caspase 3 and cleaved PARP, and attenuated the activation of JNK and AP-1. In addition, evidence suggests that ROS generated by irradiation are involved in this course of cell damage. The results indicate that BSS confers a radioprotective effect on thymocytes by regulation of the intracellular redox balance which is carried out via the scavenging of ROS and maintenance of mitochondrial membrane stability. J. Cell. Biochem. 102: 748–758, 2007.

Key words: β-sitosterol; irradiation; apoptosis; signal pathway; reactive oxygen species; radioprotection

Both radiation injury and oxidation toxicity occur after cells are exposed to ion irradiation (IR) [Dubner et al., 1995; Haimovitz-Friedman, 1998]. Oxidant toxicity results from the generation of reactive oxygen species (ROS). Cells use complex protein signaling systems to recognize radiation damage and activate various intracellular pathways that modulate cellular responses such as cell-cycle arrest, DNA repair, and apoptosis. Developments in cell biology and molecular biology continue to unravel the complexity of the molecular mechanism behind irradiation-induced cell damage.

Irradiation and ROS can damage critical cellular components such as nucleic acids, proteins, lipids, and carbohydrates. Damaged

Abbreviations used: IR, irradiation; BSS, β-sitosterol; ROS, reactive oxygen species; JNK, c-Jun NH2-terminal kinase; PARP, poly ADP-ribose polymerase; AIF, apoptosis-inducing factor; AP-1, activator protein 1; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; FCM, flow cytometer; FACS, fluorescence-activated cell sorter; ASCGE, Alkaline Single-Cell Gel Electrophoresis; FITC, fluorescein isothiocyanate; PI, propidium iodide; DCFH-DA, dichlorofluorescein-diacetate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophresis; HRP, horseradish peroxidase; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; PVDF, polyvinylidene fluoride; ECL, enhanced chemiluminescence; EDTA, ethylenediaminotetraacetic acid; NAC, N-acetylcysteine; EMSA, electrophoretic mobility shift assay; ANOVA, one-way variance analysis; SD, standard deviation; ATM, the ataxia-telangiectasia-mutated kinase; DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase.

Chun Rong Li and Zhe Zhou contributed equally to this publication.

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DNA can induce cell cycle delay or apoptosis [Radford, 1999; Marnett, 2000; Dizdaroglu et al., 2002]. The plasma and organellar membranes are also sensitive targets of irradiation [Ramakrishnan et al., 1993]. Irradiation-induced apoptotic cell death can be orchestrated by signals from the plasma membrane after association with cell surface death receptors, which is followed by caspase activation or association with ceramide. Mitochondrial membrane damage can induce mitochondrial dysfunction that not only decreases the cellular energy supply, but also triggers a change in mitochondrial potential, increases mitochondrial membrane permeability, and induces the apoptotic pathway. Mitochondria play a central role in apoptosis through caspase-dependent or caspase-independent pathways by releasing apoptogenic factors and proteases into the cytosol. These factors activate downstream caspases or death substrates in the nucleus, ultimately leading to cell death [Kim et al., 2006]. Moreover, irradiation or ROS can also alter the cytosolic signaling system and elicit a signal transduction cascade that modulates expression of apoptosis-related early expressed gene via stimulation of certain transcription factors [Dent et al., 2003a,b].

Generally, living cells have an endogenous antioxidant defense mechanism to maintain the redox balance and protect against excessive free radical action. Defenses include non-enzymatic entities such as glutathione and ascorbic acid, and the enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [Valko et al., 2001]. Radiation exposure and a high concentration of ROS can alter the balance of endogenous protective systems and lead to cell damage. Radiation resistance in many cells is presumably associated with the antioxidant enzyme system that removes ROS [Yamaguchi et al., 1994; Fang et al., 2002; Shankar et al., 2003].

Investigations on the application of radioprotective chemicals to protect normal tissues in radiation accidents or cancer therapy have been performed for several decades. A variety of radioprotective mechanisms have been proposed to explain the prophylactic and therapeutic effects of a large number of agents [Monig et al., 1990; Weiss, 1997; Maisin, 1998; Weiss and Landauer, 2000; Nair et al., 2001]. Due to the important role of ROS in the pathogenesis of several disease processes and in the initiation of a signal transduction response [Mates et al., 2000; Finkel, 2001; Martindale and Holbrook, 2002], the free radical scavenging theory is a widely accepted radiation-protective mechanism. Since antioxidants can decrease the detrimental effects of ROS production after-radiation exposure, it is feasible to develop non-toxic antioxidants as radioprotective agents.

β-sitosterol (BSS, Fig. 1) is a major phytosterol with a structural framework of 3-hydroxy cyclopentano-perhydrophenanthrene. Studies have indicated that BSS can lower cholesterol levels and has immunomodulation, anti-inflammatory, and anti-oxidative activities [Awad and Fink, 2000; Bouic, 2002; Tapieroa et al., 2003; Ovesna et al., 2004]. After finding that BSS decreases irradiation-induced thymocyte damage in vitro, this study was designed to elucidate the molecular mechanism of the radioprotective activity.

MATERIALS AND METHODS

Materials and Reagents

β-sitosterol (purity>99%) was purchased from the Chinese National Institute for Control of Pharmaceutical and Biological Products (Beijing, china). Primary antibodies (total or cleaved caspase3 and PARP, cytochrome *c*, apoptosis-inducing factor (AIF), total JNK, p-JNK, β-actin) and secondary antibody (HRP-IgG) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or Cell Signaling Technology Inc. (Danvers, MA). Annexin V-FITC-PI double-stained apoptosis detection

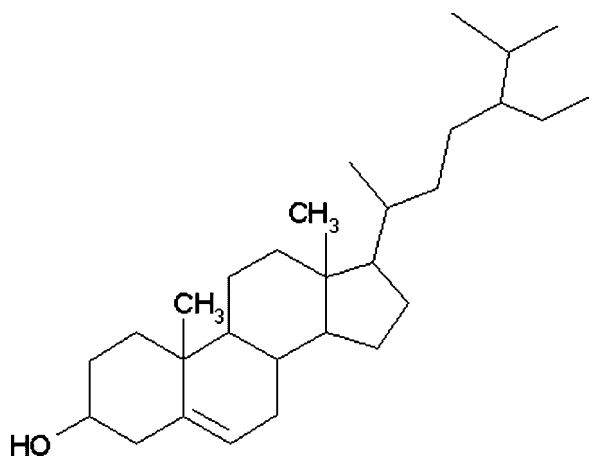


Fig. 1. Chemical structure of β-sitosterol (BSS).

kit was purchased from BaoSai biotechnology Inc. (Beijing, China).

Cell Culture and Irradiation

Thymocytes from female 4- to 6-week-old BALB/c mice (5×106/ml) were cultured routinely in RPMI-1640 medium (Invitrogen, CA)) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, MD) and 100 U/ml penicillin 100 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were incubated in 96- or 6-well plates (Costar) for tests. Irradiation was performed at room temperature with ^{60}Co γ rays source at a dose rate of 1.997 Gy/min (Beijing institute of radiation medicine).

Cell Viability

Thymocytes were plated in 96-well plates, cell viabilities were determined by the conventional the celltiter 96^R Aqueous One Solution Cell Proliferation Assay (MTS assay, Promega, Madison, WI). Thymocytes treated with or without BSS or irradiation, performed with the MTS for 2.5 h. The absorbance of the dissolved formazan was read at 490 nm using a victor^{TM} 1420 Multilabel counter (Wallac, Turku, Finland).

Cell Apoptosis

Cells were suspended in 200 μl ice-cold binding buffer and 10 μl fluorescein isothiocyanate (FITC)-labled annexinV and 5 μl propidium iodide (PI) were added in. Cell suspensions were gently mixed and incubated for 15 min at room temperature. Apoptosis was determined by flow cytometry (FCM, FACSCalibur, B-D Company). Annexin V positive and PI negative cells were defined as apoptotic cells. Both Annexin V-FITC and PI negative cells were considered as viable cells, while both Annexin V-FITC and PI positive cells were considered as late apoptotic or already dead cells.

Intracellular ROS Assessment

The fluorescent probe DCFH-DA (Sigma) was used to measure intracellular ROS production with fluorescence-activated cell sorter (FACS) analyses. Thymocytes (5×10^6 cells/ml) suspensions loaded with probe were incubated at 37°C for 20 min, and cells were harvested and rinsed with RPMI-1640 medium sufficiently. ROS was determined by flow cytometer (excitation and emission wavelengths were 485 and 535 nm) and assessed by average fluorescence intensity.

ASCGE (Alkaline Single-Cell Gel Electrophoresis, Comet Assay)

Single cell gel electrophoresis was performed under alkaline conditions as previously described with minor modifications [Collins, 2004; Moller, 2005]. Briefly, the slides were pretreated with agar. Fifty microliters of the cell suspensions was mixed with 50 μl of 1% low melting point agarose. Mixtures were spread onto microscope slides pre-coated with 0.7% high melting point agarose, another 50 μl of 0.7% low melting point agarose was covered on the surface. The slides were kept in 4°C refrigerator for 20 min for agroase solidified and then immersed in chilled lysis buffer (containing 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris base pH 10, 1% sarcosine Na, 1% Triton X-100, and 10% DMSO) at 4°C for 1.5 h. Transferred them into a horizontal electrophoresis tank with chilled electrophoresis buffer (30 mM- NaOH, 1 mM Na_2-EDTA) for 20 min to allow DNA unwinding, and then performed electrophoresis at 25 V and 200 mA at 4°C for 20 min. After that, the slides were placed in a neutralizing buffer (0.4 M Tris–HCl, pH 7.5) at 4°C for 15 min and stained with PI. Pictures of the comets were obtained with a Laser Scanning Confocal Microscopy (Bio-Rad Radiance 2100). One hundred comets were observed of each sample. Intensity of the comet tail DNA relative to the head DNA reflects the number of DNA breaks was determined with comet assay software project v.1.2.2.

Assay for Antioxidant Enzymes

Cells were harvested by centrifugation and resuspended in 1 ml lysis buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.02% NaN_3; 100 μg/ml PMSF; 1 μg/ml aprotinin; 1% Triton X-100) 30 min at 4°C, then enzyme assays were carried out. Total protein was measured by the Bradford method. SOD activity was assayed by the reported method [Kakkar et al., 1984]; CAT activity was assayed by the procedure of Sinha [1972]; activity of GPx was assayed by the method of Rotruck et al. [1973].

Western Blotting Analysis

Cell extracts were prepared using relevant kits (cell extraction kit, nuclear-cytosol

extraction kit and mitochondria/cytosol isolation kit) which was purchased from Applygen Technologies Inc. (Beijing, China). Concentrations of proteins were quantified by the Bradford method. Western blotting assay was done according to the described method [Yakunin and Hallenbeck, 1998]. In brief, equal amounts of protein were separated on sodium-dodecyl sulfate-polyacrylamide (SDS–PAGE) gel and transferred to a polyvinylidene fluoride (PVDF) membrane (Schleicher and Schuell Biosciences, Keene, NH). Membranes were blocked for 2 h at room temperature with 5% (w/v) non-fat dried milk in TBS-Tween-20 (0.05%) and incubated with specific primary antibodies for 2 h and the appropriate second antibody HRP-IgG for 1 h at room temperature, orderly. Immuno-reactive protein bands were detected with the enhanced chemiluminescence (ECL) Western blotting detection system (Santa Cruz Biotechnology Inc.), and visualized by exposing to X-ray film.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed according to the described method with slight modification [Chaturvedi et al., 2000]. Nuclear protein were extracted using extraction kit from Applygen Technologies Inc. and quantified by the Bradford method .The DNA binding activity of transcription factors was assayed according to the manufacturer's instructions (Pierce Inc.). In brief, HRP-labeled oligonucleotide primers containing AP-1 binding site (5′-CGC TTG ATG AGT CAG CCG GAA-3′; 3′-GCG AAC TAC TCA GTC GGC CTT-5′) were prepared at the concentration of 0.5 μg/μl, 10 μg of nuclear protein was incubated in gel shift binding buffer on ice for 10 min, and then co-incubate with the primers for 20 min. The DNA–protein-binding complex was run on a 6% non-denatured polyacrylamide gel at 250 V, and then transferred to a PVDF membrane. The membrane were blocked for 15 min with blocking buffer and incubated for 1 h at room temperature with horseradish peroxidase-conjugated streptavidin (Vector Inc.). The specific bands were detected with the ECL and visualized by exposing to X-ray film.

Statistics

All experiments were performed at least three times. Statistical calculations were done using SPSS 13.0 software with one-way variance analysis (ANOVA). Results were expressed as means ± standard deviation (SD). Significance levels were defined as $P < 0.05$.

RESULTS

Pretreatment With BSS Protected Thymocyte Against Irradiation-Induced Apoptotic Death

Thymocytes pretreated with or without BSS were exposed to 6 Gy irradiation. Cell apoptosis was detected 6 h postirradiation and cell viability was detected 24 h postirradiation. As shown in Figure 2 BSS (2.4 μM) attenuated the reducing of cell viability by irradiation at dose of 2–6 Gy and had no cytotoxicity (data not shown) and played in concentration-dependant manner (Fig. 4). Apoptosis was the main form of irradiation-induced thymocyte early death and increased in a dose-dependant manner in irradiated thymocytes (Fig. 3A). Pretreatment of thymocytes with BSS at the concentration of 0.024 or 2.4 μM decreased irradiation-induced apoptosis in the early damaged course (Fig. 3B).

BSS Reduced Irradiation-Induced Nuclear DNA Strand Break

DNA damage is principal event in irradiated cells and DNA double-stand break is the most important form of a series of DNA damages for cell killing. Thymocytes pretreated with or without BSS were exposed by 6 Gy irradiation. DNA double-stand breaks were detected by

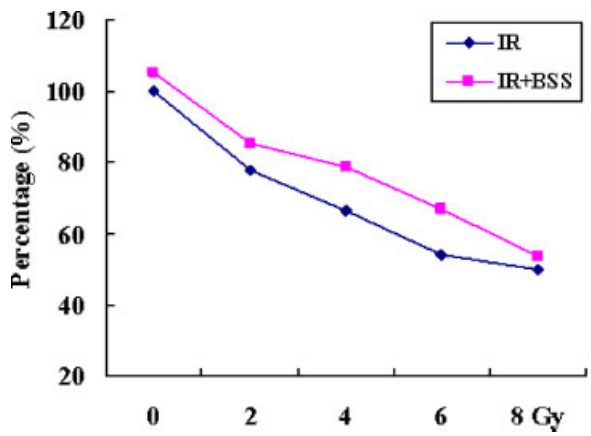


Fig. 2. Effects of irradiation and BSS on thymocytes survival. Thymocytes (5×10^5/well) were cultured in 96-well plates with or without BSS (0.024–2.4 μM) and exposed to different doses of irradiation. Cell viabilities were measured by MTS assay and expressed as a percentage of absorbance seen in the untreated control cells which indicated the relative number of cells survived in irradiation damage. Each value represents the means ± SD. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

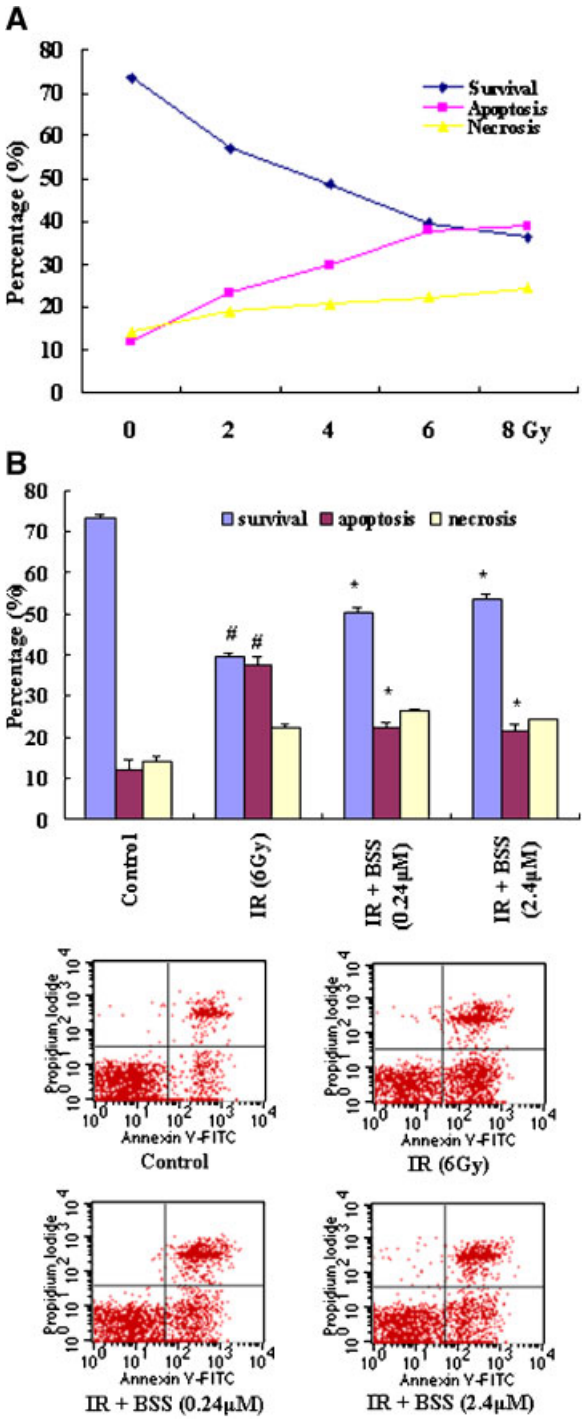


Fig. 3. The effect of BSS on irradiation-induced thymocye apoptosis. **A**: Thymocytes were exposed to different dose of irradiation. **B**: Thymocytes pretreated with or without BSS (at concentration of 0.24 or 2.4 μM) exposed to 6 Gy irradiation. Cells in (A) and (B) above were harvested and labeled with both Annexin V-FITC and PI. Apoptosis were confirmed by flow cytometer. The percentages of apoptotic cells were compared. Each value represents the means ± SD ($^{\#}P < 0.05$ shows significantly different from control; $^{*}P < 0.05$ shows significantly different from irradiated cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

alkaline single cell gel electrophoresis 3 h postirradiation. Figure 4 shows that BSS present at 0.24 or 2.4 μM reduced irradiation-induced nuclear DNA strand break.

BSS Regulated Intracellular ROS

Given that antioxidant character of BSS is reported, so we supposed that the BSS protective effect may be relative to its scavenging ability on ROS. We detected intracellular ROS through flow cytometer. It was observed that exposure to 6 Gy irradiation result in marked increasing of ROS in thymocyte, and it showed lower content of ROS in irradiated thymocyte which pretreated with BSS (Fig. 5).

BSS Increased the Activation of Antioxidant Enzyme in Irradiated Thymocyte

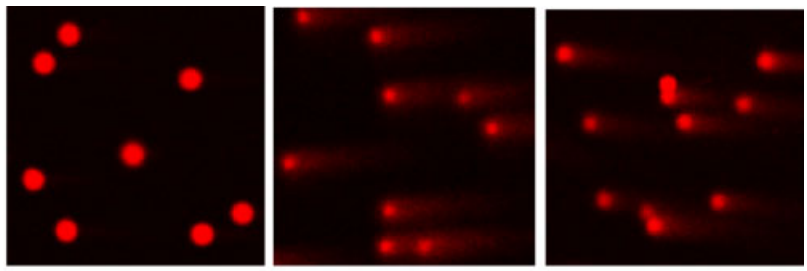
Antioxidant enzyme is essential to keep the intracellular redox balance especially when high concentration of ROS exists in cells. The thymocytes pretreated with BSS were exposed to irradiation. Cell total proteins were harvested for activity assay. Presence of BSS in irradiated thymocyte increased the antioxidant enzyme activities of SOD, CAT, GPx in comparison with that of irradiation treatment alone. It showed us that the modulating effects of BSS on antioxidant enzymes activities (Fig. 6).

BSS Inhibited the Releasing of Apoptosis-Related Protein From Mitochondrial Intermembrane Space

We also evaluated change of mitochondria membrane permeability in irradiated thymocyte with or without BSS by detecting release of cytochrome *c*, AIF which is important intermembrane protein essential to energy conservation and involved in mitochondria-related apoptotic pathway when release from mitochondria. As shown in Figure 7, pretreatment of thymocyte with BSS partly inhibited the irradiation-induced releasing of cytochrome and mitochondria-nuclear translocation of AIF. Similar result was found in thymocyte co-treated with N-acetylcysteine (NAC, ROS scavenger) and irradiation, hinted those proteins release involve in ROS.

The Effects of BSS on Irradiation-Induced Increase in Cleavage of Caspase 3

Caspase 3 is a primary executor in the apoptotic pathways. Activation of caspase 3



Effect of BSS on irradiation-induced cell damage

Samples	Cell viablily (MTS,%)	DNA damage (Tail-DNA,%)
Control	100.00±0.00	0.05±0.01
IR (6Gy)	52.65±1.24#	27.17±1.38#
IR + BSS (0.024μM)	57.92±1.89	24.07±0.46
IR + BSS (0.24μM)	59.16±0.95*	23.96±1.00*
IR + BSS (2.4μM)	62.67±1.13*	21.44±0.69*

Values are given as mean ± S.D, differ significantly at $p < 0.05$
#, compared to control; *, compared to IR

Fig. 4. The effect of BSS on irradiation-induced nuclear DNA strand break. Thymocytes pretreated with or without BSS (0.24 or 2.4 μM) exposed to 6 Gy irradiation. Cells were harvested 3 h postirradiation and DNA strand break was detected through alkaline single cell gel electrophoresis. The percentages of Tail-DNA were compared. Each value represents the means ± SD ($^{\#}P < 0.05$ shows significantly different from control; $^{*}P < 0.05$ shows significantly different from irradiated cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

occurs in response to variety of apoptotic inducers. Releasing of cytochrome *c* may lead to cleavage of caspase 3 through activating caspase 9. We evaluated the influence of BSS on caspase 3 activation to ascertain whether radioprotection of BSS is correlated with this caspase cascade apoptotic pathway. Figure 8A shows the changes in levels of cleaved caspase 3 induced by irradiation or/and BSS. Irradiation increased cleavage of caspase 3, and BSS reduced the radiation-induced increase in levels of cleaved caspase 3. Total caspase 3 was not affected by either irradiation treatment or BSS.

The Effects of BSS on Irradiation-Induced Increase in Cleavage of PARP

PARP, a 116 kDa nuclear poly ADP-ribose polymerase, is one of main cleavage target of caspase 3. Cleavage of PARP (85 kDa) facilitates cellular disassembly and serves as a maker of cells undergoing apoptosis. We evaluated the levels of cleaved PARP in irradiated thymocytes which pretreated with or without BSS. Similar to the changes seen with caspase 3, irradiation treatment enhanced cleavage of PARP and pretreatment with BSS prevented irradiation-induced changes in PARP cleavage. The levels of total PARP were unaffected by either irradiation treatment or presence of BSS (Fig. 8B).

BSS Attenuated the Activation of JNK and AP-1

We studied the effects BSS on the *c*-Jun NH2-terminal kinase (JNK) pathway which is a key modulator in cell apoptotic death. The results demonstrate the change in the levels of phosphorylated JNK in irradiated thymocytes which pretreated with or without BSS. As shown in Figure 9A, irradiation treatment (6 Gy) induced increase in JNK phosphorylation. The presence of BSS or NAC partly inhibited the radiation-induced phosphorylation of JNK. Neither radiation treatment nor the addition of

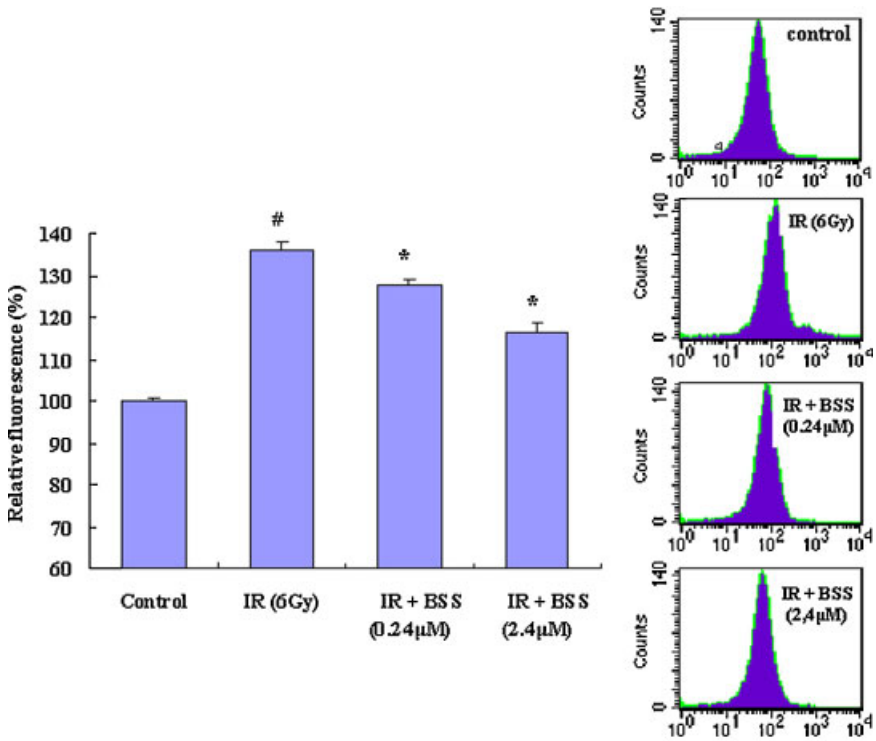


Fig. 5. The influence of BSS on intracellular ROS level in irradiated thymocyte. Thymocytes pretreated with or without BSS (0.24 or 2.4 μM) exposed to 6 Gy irradiation. Cells were harvested 1 h postirradiation and treated with DCFH-DA (10 μM). Intracellular ROS levels were determined by measuring DCF fluorescence which was detected by flow cytometer and relative fluorescence intensities were compared, the control cell was considered 100%. ($^{\#}P < 0.05$ shows significantly different from control; $^{*}P < 0.05$ shows significantly different from irradiated cells). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

BSS or NAC caused any change in the total levels of JNK protein. Inhibitory effect of NAC hinted the activation of JNK is related to ROS. Furthermore, we also detected activation of activator protein 1 (AP-1, a transcription fact as the downstream of JNK) according to its binding ability with relevant DNA sequence. Figure 9B shows the presence of BSS partly inhibited the radiation-induced activation of AP-1.

Effect of BSS on activities of antioxidant enzymes in irradiated thymocytes

Activities of antioxidant enzymes	SOD (50% inhibition of NBT reduction/min/mgprot)	CAT (μmol of H_2O_2 consumed/min/mgprot)	GPx (μg of GSH consumed/min/mgprot)
Control	33.10±1.62	9.90±0.46	18.88±0.93
IR (6Gy)	21.58±0.93#	4.21±1.01#	9.67±0.76#
IR+BSS(0.24 μM)	41.78±1.11*	8.88±0.29*	15.78±1.04*
IR+BSS(2.4 μM)	46.33±2.10*	9.01±0.33*	13.26±0.69*

Values are given as mean ± S.D. (n = 6).differ significantly at p < 0.05

#, compared to control; *, compared to IR

Fig. 6. The effect of BSS on activities of antioxidant enzymes in irradiated thymocyte. Thymocytes pretreated with or without BSS (0.24 or 2.4 μM) exposed to 6 Gy irradiation. Whole cell proteins were harvested 3 hour postirradiation and enzyme assays were carried out. ($^{\#}P < 0.05$ shows significantly different from control; $^{*}P < 0.05$ shows significantly different from irradiated cells).

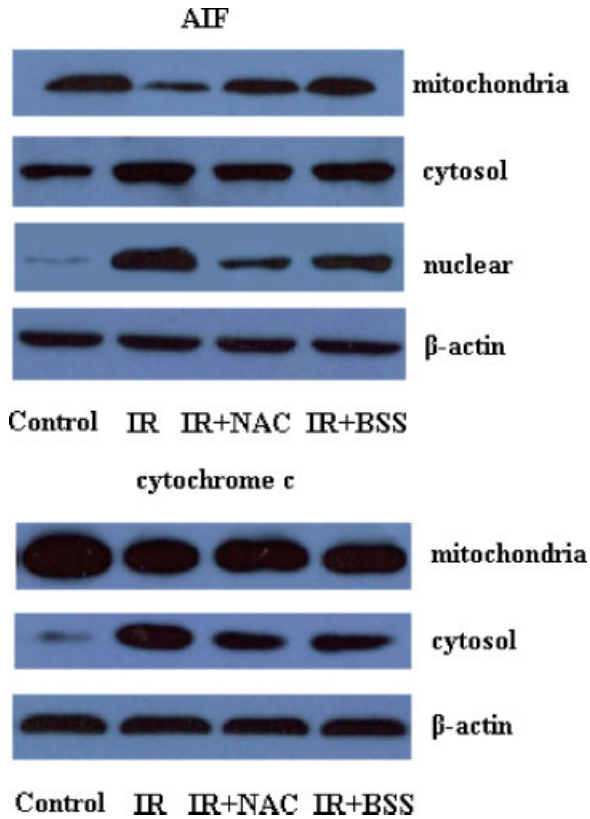


Fig. 7. The effect of BSS on releasing of mitochondria intermembrane protein in irradiated thymocyte. Thymocytes pretreated with BSS (2.4 μM) or NAC (10 mM) were exposed to 6 Gy irradiation and mitochondrial, cytoplasmic or nuclear extracts were collected 3 h postirradiation. Levels of cytochrome *c* and AIf were determined using Western blotting. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

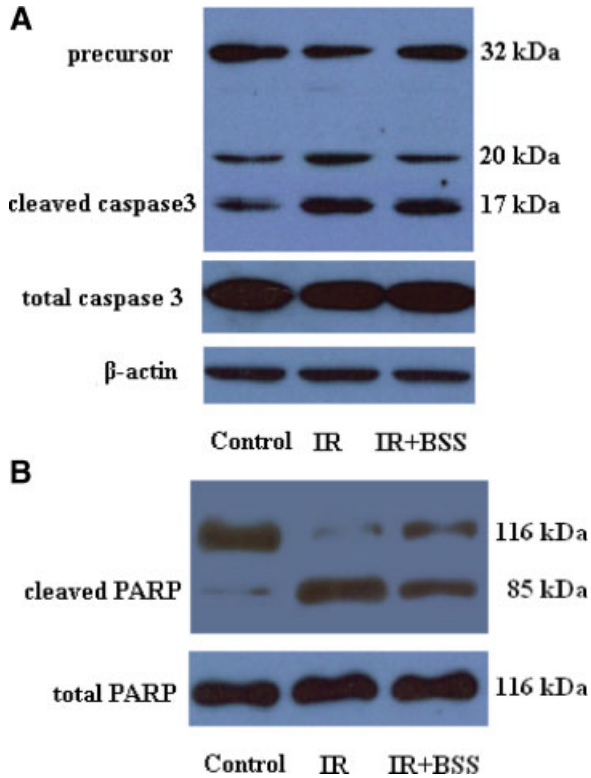


Fig. 8. The effects of BSS on levels of cleaved caspases 3 and PARP in irradiated thymocyte. **A**: Thymocytes pretreated with BSS (2.4 μM) were exposed to 6 Gy irradiation and whole cell extracts were collected 3 h postirradiation. **B**: Thymocytes pretreated with BSS (2.4 μM) were exposed to 6 Gy irradiation and whole cell extracts were collected 6 h postirradiation. Levels of cleaved caspases 3 and PARP were determined using Western blotting assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

This investigation focused on the possible role of BSS as a protector against irradiation-induced thymocyte early damage and irradiation-mediated activation of apoptotic pathways. Thymocytes were chosen to study the effects of BSS because this is the cell type most sensitive to irradiation and they play an important role in immune system function. In this experiment, BSS decreased irradiation-induced thymocyte apoptosis and DNA damage while attenuating intracellular ROS, which increased significantly after cellular irradiation. Since the generation of ROS by irradiation is a noteworthy mediator of cell damage, it is a reasonable supposition that the radioprotection provided by BSS might be associated, at least in part, with its antioxidant activity by scavenging ROS. It was actually found that BSS increases the activities of antioxidant enzymes such as SOD, CAT, and GPx, which are decreased in irradiated cells. The ability to enhance antioxidant activity benefits the ROS-scavenging action of BSS and helps maintain the redox balance in cells. Further work was performed to determine a possible molecular mechanism for the decrease in apoptotic death after BSS treatment.

Apoptosis is the main form of irradiation-induced thymocyte early death, and multiple signaling pathways activated by irradiation mediate apoptosis in a cell-type-dependent manner [Verheij and Bartelink, 2000; Cho and Choi, 2002; Dent et al., 2003a,b]. It is widely known that DNA damaged by irradiation mediates cell cycle delay or apoptosis once lesions are recognized by enzymes such as the ataxia-telangiectasia-mutated kinase (ATM), the catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), PARP, and p53 [Ko and Prives, 1996; Canman et al., 1998; Szumiel,

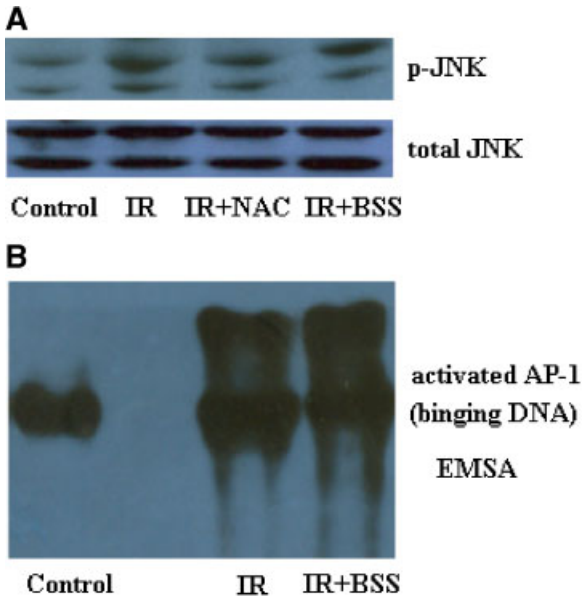


Fig. 9. The inhibitory effects of BSS on phospho JNK levels and activation of AP-1 in irradiated thymocytes. **A**: Thymocytes pretreated with BSS (2.4 μM) were irradiated (6 Gy) and whole cell extracts were collected 1 h postirradiation. Levels of phosphorylated JNK were determined using western blotting assay. **B**: Nuclear extracts prepared from thymocytes co-treated with irradiation and or without BSS 6 h postirradiation. AP-1 activation was determined by electrophoretic mobility shift assay. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1998; Zhang et al., 2002; Yang et al., 2003; Burma and Chen, 2004; Kurz and Lees-Miller, 2004]. In a series of DNA lesions, strand break are the most important inducers of cell killing. The decrease in strand break in irradiated thymocytes treated with BSS not only maintains gene stability, but as a result might also reduce apoptotic death stimulated by damaged DNA. Radiation-induced DNA damage is caused directly by ionization of DNA or indirectly by the action of ROS generated by the ionization of water molecules. Therefore, BSS might decrease DNA damage by scavenging ROS.

Radiation-induced apoptosis can also be initiated at the mitochondrial level. Membrane damage caused by irradiation or ROS results in a permeabilization of the mitochondrial outer membrane, causing the release of several proteins—cytochrome *c*, Smac/Diablo, AIF, endonuclease G, and Omi/HtrA2—that play important roles in various aspects of the cell death process [Li et al., 1997; Li et al., 2001; Cande et al., 2002; van Gurp et al., 2003]. Cytochrome *c* binds and activates apoptotic protease activating factor (Apaf)-1 to recruit and activate caspase-9 and initiates the caspase cascade apoptotic process. In this experiment, BSS decreased the release of cytochrome *c* from the mitochondrial intermembrane to the cytosol while affecting a concomitant partial inhibition of radiation-induced increases in cleaved caspase 3 and cleaved PARP. Caspase 3 is an apoptotic caspase that acts as a downstream substrate of caspase 9, while PARP is a substrate of caspase 3 that function in DNA repair that be inactivated by caspases during the execution phase of apoptosis. These results suggest that a caspase-related apoptotic pathway is partially inhibited by treatment of thymocytes with BSS prior to irradiation. Furthermore, BSS was also found to inhibit the irradiation-induced translocation of AIF. As reported, mitochondrio-nuclear translocation of AIF and EndoG might initiate chromatin condensation and large scale DNA fragmentation, and could be associated with a caspase-independent apoptotic pathway that is involved in irradiation-induced cell death. The cumulative findings suggest that pretreatment with BSS helps maintain the integrity of the mitochondrial membrane, thereby decreasing cell apoptosis or DNA damage. NAC is an antioxidant which has been widely used as tool for investigation the role of ROS in numerous biological and pathological processes. In our experiment, NAC inhibited the release of cytochrome *c* and the mitochondrio-nuclear translocation of AIF, hinting that ROS generated by irradiation are involved in the course of cell damage and that BSS reduces mitochondria-related apoptosis by scavenging those ROS.

In various cell systems, the damage response to ionizing radiation involves activation of the stress-activated protein kinase or *c*-Jun N-terminal kinase (SAPK/JNK) signaling pathway. JNK is an important kinase in the cytosol, primarily acting downstream of the death receptors, and can also be activated by ROS. JNK activates transcription factors that regulate the expression of a number of apoptosis-related genes [Chen et al., 1996; Verheij et al., 1998; Shena and Liub, 2006]. BSS partly inhibited activation of JNK and its downstream transcription factor AP-1 in irradiated thymocytes, thereby reducing expression of apoptosis-related genes and protecting cells against further apoptotic activity. Pretreatment with

ROS scavenger NAC also partially inhibited activation of JNK, again suggesting that ROS are involved in the damage.

Bcl-2 and Bax are apoptosis-related proteins that act to preserve the stability of the mitochondrial membrane [Yang and Korsmeyer, 1996; Tsujimoto, 2003; Er et al., 2006]. Activated JNK phosphorylates Bcl-2 to weaken its protective effect on mitochondrial membrane or phosphorylates Bim-related members of the Bcl-2 family to induce Bax-dependent apoptosis [Lei and Davis, 2003]. They provide a molecular link between the JNK signal transduction pathway and the mitochondrial apoptotic pathway and imply a possible radioprotective mechanism for BSS in regulating Bcl-2 family proteins and maintaining a functional mitochondrial membrane.

In conclusion, BSS plays an important role in regulating irradiation-induced early cell damage via the scavenging of ROS and maintenance of mitochondrial membrane stability. The potential of antioxidants to reduce the cellular damage induced by ionizing radiation has been studied for many years. Some phytochemicals exhibit antioxidant characteristics and most of them are non-toxic or have a low toxicity compared with most synthetic protectors. Therefore, they are a promising source of radioprotective agents [Weiss and Landauer, 2003]. BSS is abundant in plant oils and is an active component in many Chinese traditional medicines. A safety evaluation showed that BSS has neither genotoxic nor cytotoxic effects on a mouse model, even at doses of 200–1000 mg/kg [Paniagua-Pérez et al., 2005]. This study indicates the possibility of the use of BSS as a radioprotector or accessorial agent to radioprotection.

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