

Ginsenoside Rg3 induces DNA damage in human osteosarcoma cells and reduces MNNG-induced DNA damage and apoptosis in normal human cells

YUE-HUI ZHANG^{1*}, HAI-DONG LI^{1,2*}, BO LI¹, SHENG-DAN JIANG¹ and LEI-SHENG JIANG¹

¹Department of Orthopedic Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200092;

²Department of Orthopedic Surgery, First People's Hospital, Huzhou Normal Institute, Zhejiang 313000, P.R. China

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Abstract. *Panax ginseng* is a Chinese medicinal herb. Ginsenosides are the main bioactive components of *P. ginseng*, and ginsenoside Rg3 is the primary ginsenoside. Ginsenosides can potently kill various types of cancer cells. The present study was designed to evaluate the potential genotoxicity of ginsenoside Rg3 in human osteosarcoma cells and the protective effect of ginsenoside Rg3 with respect to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced DNA damage and apoptosis in a normal human cell line (human fibroblasts). Four human osteosarcoma cell lines (MG-63, OS732, U-2OS and HOS cells) and a normal human cell line (human fibroblasts) were employed to investigate the cytotoxicity of ginsenosides Rg3 by MTT assay. Alkaline comet assay and γ H2AX focus staining were used to detect the DNA damage in MG-63 and U-2OS cells. The extent of cell apoptosis was determined by flow cytometry and a DNA ladder assay. Our results demonstrated that the cytotoxicity of ginsenoside Rg3 was dose-dependent in the human osteosarcoma cell lines, and MG-63 and U-2OS cells were the most sensitive to ginsenoside Rg3. As expected, compared to the negative control, ginsenoside Rg3 significantly increased DNA damage in a concentration-dependent manner. In agreement with the comet assay data, the percentage of γ H2AX-positive MG-63 and U-2OS cells indicated that ginsenoside Rg3 induced DNA double-strand breaks in a concentration-dependent manner. The results also suggest that ginsenoside Rg3 reduces the extent of MNNG-induced DNA damage and apoptosis in human fibroblasts.

Introduction

Osteosarcoma is a malignant bone tumor that usually develops in adolescents and young adults. The National Cancer Institute estimates the number of new cases of osteosarcoma each year to be 4.4 per one million among individuals aged 0-24 years (1). Most treatment protocols for osteosarcoma use an initial period of systemic chemotherapy prior to definitive resection of the primary tumor. Notably, most of the commonly used chemotherapeutic drugs kill normal as well as cancer cells (2). Therefore, drugs targeted specifically at cancer cells while leaving normal cells intact, or even protecting normal cells, would be ideal for use as part of a chemotherapy regimen.

Panax ginseng was discovered over 5,000 years ago in the hills of Manchuria in China. Since then, the plant has held its place as a highly venerated medicinal plant in traditional Chinese medicine (3). Over recent decades, the anticancer effects of ginsenosides have garnered increasing attention because of their favorable safety and efficacy profiles (4). Ginsenosides enhance cytotoxic and humoral immune responses (5). Recent research has shown that ginsenosides can inhibit the growth of several cancer cell lines (6). The anticarcinogenic and antimetastatic effects of ginsenoside Rg3 have been demonstrated *in vitro* and *in vivo* (7). Ginsenoside Rg3 induces cell cycle arrest and apoptosis in mammalian tumor cells (8). However, few published reports describe the genotoxicity of ginsenoside Rg3. In contrast, the cytoprotective effect of ginsenosides has been demonstrated in other studies. Poon *et al* (9) showed that ginsenoside 20-Rg3 protected against BaP-induced DNA damage in human dermal fibroblasts (HDFs). Ginsenoside Rg3 was found to protect against cyclophosphamide-induced DNA damage and cell apoptosis by reducing oxidative stress (10). Ginseng extract was also found to protect against γ -ray-induced DNA double-strand breaks (11).

The results outlined above suggest that ginsenosides could be used for the chemotherapeutic treatment of patients with osteosarcomas. Theoretically, ginsenosides would destroy cancer cells but leave normal cells unharmed. To prove our hypothesis, we explored the effects of ginsenoside Rg3 on *in vitro* DNA damage and apoptosis in human osteosarcoma cell lines. The presumed cytoprotective effect of ginsenoside

Correspondence to: Professor Lei-Sheng Jiang, Department of Orthopedic Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, P.R. China

E-mail: jiangleisheng@126.com

*Contributed equally

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Rg3 was investigated in human fibroblasts. Sensitive and quantitative detection assays (e.g., the alkaline comet assay, measurements of γ H2AX focus formation, flow cytometry and DNA ladder assay) were used. The results confirmed that ginsenoside Rg3 exhibited obvious genotoxicity against human osteosarcoma cells and protected normal human cells against MNNG-induced DNA damage and apoptosis.

Materials and methods

Chemicals and reagents. Ginsenoside Rg3 (purity, >96%) was purchased from Tianping Pharmaceutical Co., Shanghai, China. The compound was dissolved in dimethyl sulfoxide (DMSO). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), trypan blue, low-melting agarose (LMA), normal-melting agarose (NMA), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), 4',6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Tween-20 and paraformaldehyde were obtained from Sigma Chemical Co. (Silicon Valley, CA, USA). An apoptosis detection kit was obtained from BD Pharmingen. Triton X-100, ethidium bromide (EtBr), fetal bovine serum (FBS), xylene cyanol, and bromophenol blue were obtained from Sangon Biotech Shanghai Co., Ltd. (Shanghai, China). Propidium iodide (PI) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (Grand Island, NY, USA). Other common chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Cell culture. The human osteosarcoma MG-63, OS732, U-2OS and HOS cell lines and human fibroblasts were purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100 U/ml). Cells were incubated at 37°C in a 5% CO₂ incubator. The medium was exchanged once every 2 days. After treatment, the cells were harvested by trypsinization.

Cell viability test. Cytotoxicity was measured using the MTT assay (12). The trypan blue dye exclusion assay was performed to confirm and verify cell viability. The human osteosarcoma cells and fibroblasts were seeded at a density of 1×10^4 cells/well in 100 μ l of the cell culture medium, and then placed into a 96-well plate. After 12 h of incubation, the cells were treated with 0–300 μ M ginsenoside Rg3 for 24 h. Then, MTT solution (5 mg/ml) was added to each well, and the samples were incubated at 37°C for 4 h. Thereafter, the supernatant was removed and replaced with 100 μ l of DMSO. Optical density (OD) of the control and drug-treated wells was measured using an automated microplate reader (Multiskan EX; Lab Systems, Vantaa, Finland) at a test wavelength of 570 nm.

Alkaline comet assay. The alkaline comet assay was performed according to the procedure described by Calderon-Segura *et al* (13), with slight modifications for the evaluation of DNA single-strand breaks (SSBs). Cells were cultured in each well of a 24-well plate, at a density of 1×10^5 /ml. Then,

MG-63 and U-2OS cells were treated with 0, 25, 50, 100 and 150 μ M ginsenoside Rg3 for 24 h. The cells treated by MNNG (20 μ M) were used as the positive control and the cells treated by 0 μ M ginsenoside Rg3 were used as the negative control in the genotoxicity study. Human fibroblasts were treated with ginsenoside Rg3 (50 mM) and MNNG (20 mM) for 24 h. Then, cells were collected, washed, and suspended in PBS (pH 7.4); 30- μ l cell samples (1×10^4 cells) were used and suspended in 110 μ l of 1% molten LMA at 37°C. The mono-suspension was cast on a microscopic slide covered with a layer of 0.8% NMA. The agarose was gelled at 4°C, after which the slides were immersed in a fresh lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% Triton X-100, 10% DMSO and pH 10.0) for 30 min at 4°C. After lysis, the slides were washed in distilled water three times and immersed in fresh alkaline electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13.0) for 10 min at 4°C. An electric field was then applied at 20 V (1 V/cm) and 300 mA for 10 min. The slides were neutralized to pH 7.5 in 0.4 mM Tris buffer and stained with 40 μ l of 20 μ g/ml EB. The signal emitted was analyzed using an Olympus BX53 fluorescence microscope (Olympus, Tokyo, Japan) with a 515- to 560-nm filter. The extent of DNA migration was determined using an image analysis system (CASP, www.casp.of.pl). Parameters such as tail length (DNA migration from the nucleus), tail DNA (DNA content in the tail), and the tail moment (migrated DNA in the tail multiplied by the tail length) were recorded.

γ H2AX focus staining. The phosphorylation of histone H2AX was used as a marker of DNA double-strand breaks with slight modifications (14). MG-63 and U-2OS cells (1×10^5) were seeded onto 6-well culture plates and treated with 0, 25, 50 and 100 μ M of ginsenoside Rg3 and 20 μ M MNNG for 24 h. Human fibroblasts were treated with ginsenoside Rg3 (50 mM) and MNNG (20 mM) for 24 h. After treatment, the cells were fixed in 4% paraformaldehyde for 15 min, washed with PBST (PBS buffer pH 7.4 and 0.1% Tween-20), and permeabilized in 1% Triton X-100 for 30 min. After blocking the cells with serum for 60 min, the samples were incubated with a rabbit monoclonal anti- γ H2AX antibody (1:1,500; Cell Signaling Technology) overnight at 4°C, and then incubated with an Alexa594-conjugated anti-rabbit secondary antibody (1:360; Cell Signaling Technology) for 60 min. To stain the nuclei, cells were incubated in DAPI (1 mg/ml) for 15 min. The cells were then mounted in Antifade media, and images were captured using an Olympus BX53 fluorescence microscope (Olympus). The objectives were set at wavelengths of 594 nm for γ H2AX and 350 nm for DAPI.

DNA extraction and detection of DNA fragmentation. The DNA ladder assay was performed according to the protocol published previously by our own laboratory. After the cells were treated with ginsenoside Rg3 and MNNG at concentrations of 50 and 20 μ M, respectively, for 24 h, pellets containing 1×10^6 cells were lysed in lysis buffer (10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 100 mM NaCl and 400 g/ml protease K) for 120 min at 56°C, then treated with 10 mg/ml RNase A for an additional 50 min at 37°C. The lysates were centrifuged (12,000 $\times$ g for 30 min at 4°C), and the supernatant was collected. The fragmented

DNA was extracted from the supernatant with a neutral phenol:chloroform:isoamyl alcohol mixture (v/v/v, 25:24:1). The DNA pellet was precipitated by adding isopropanol, then washed with 75% ethanol, and dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA fragmentation was detected by electrophoresis through an agarose gel, and the bands were stained with ethidium bromide for UV light visualization.

Detection of apoptotic incidence by flow cytometry. Apoptotic incidence was measured using the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's instructions. Briefly, cells were treated with ginsenoside Rg3 and MNNG at concentrations of 50 and 20 μ M, respectively, for 24 h. Then, cells were washed twice with cold PBS and resuspended in 500 μ l of binding buffer at a concentration of 1×10^6 cells/ml. Then, 5 μ l of Annexin V-FITC solution and 5 μ l of propidium iodide (PI; 1 mg/ml) were added; cells were incubated at 37°C for 30 min. The cells were analyzed by flow cytometry within 1 h. Apoptotic cells were counted and represented as a percentage of the total cell count.

Statistical analysis. The data are expressed as the mean values ($\pm$ SEM) of 3 independent experiments. The differences among the treated groups and the negative control were compared by one-way analysis of variance. The Newman-Keuls multiple comparisons test was applied; the significance level was set at $P < 0.05$. All statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

Cytotoxic effects of ginsenoside Rg3. To identify the cytotoxic effect of ginsenoside Rg3 on human osteosarcoma MG-63, OS732, U-2OS and HOS cell lines and fibroblasts, we initially treated these cells with various concentrations of ginsenoside Rg3 for 24 h. Cell viability was estimated by MTT assay and trypan blue staining. Ginsenoside Rg3 triggered a concentration-dependent decrease in the viability of MG-63 and U-2OS cells. According to our results, MG-63 and U-2OS cells were the most sensitive to ginsenoside Rg3 and, thus, the two cell lines were used in the subsequent experiments. Meanwhile, ginsenoside Rg3 had no effect on the cell viability of the normal human cells (human fibroblasts) (Fig. 1).

Alkaline comet assay for the identification of DNA single-strand breaks. When subjected to the alkaline comet assay, DNA fragments migrate to form a comet-like image. For the negative control, comet heads contained high-density DNA and exhibited smooth margins and intact nuclei. MG-63 and U-2OS cells accounted for 6% of those in comet-like formations. In the groups treated with ginsenoside Rg3, DNA comets exhibited broom-shaped tails. The fluorescence intensity of their heads was weaker than that of the negative control (Fig. 2A). After treatment with ginsenoside Rg3, the percentages of comet-positive MG-63 and U-2OS cells were significantly increased ($P < 0.01$) at 50, 100 and 150 mM, when compared to the negative control (Fig. 2B).

The characteristics of MG-63 and U-2OS cells exposed to ginsenoside Rg3, including mean ($\pm$ SEM) tail length, tail

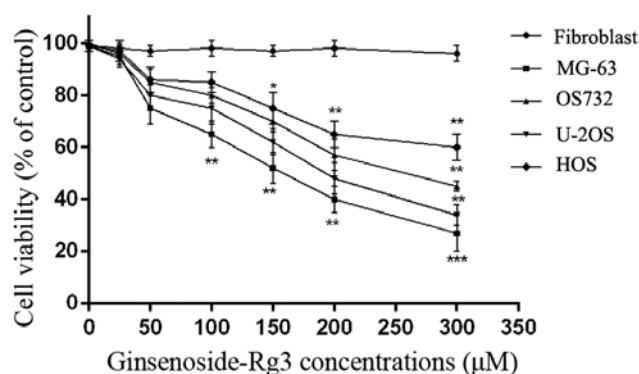


Figure 1. Cytotoxicity of ginsenoside Rg3 in human osteosarcoma MG-63, OS732, U-2OS and HOS cell lines and fibroblasts. Cell viability was determined by MTT assay and expressed as the mean values ($\pm$ SEM) of 3 independent experiments. * $P < 0.05$ and ** $P < 0.01$ represent significant differences relative to the negative controls.

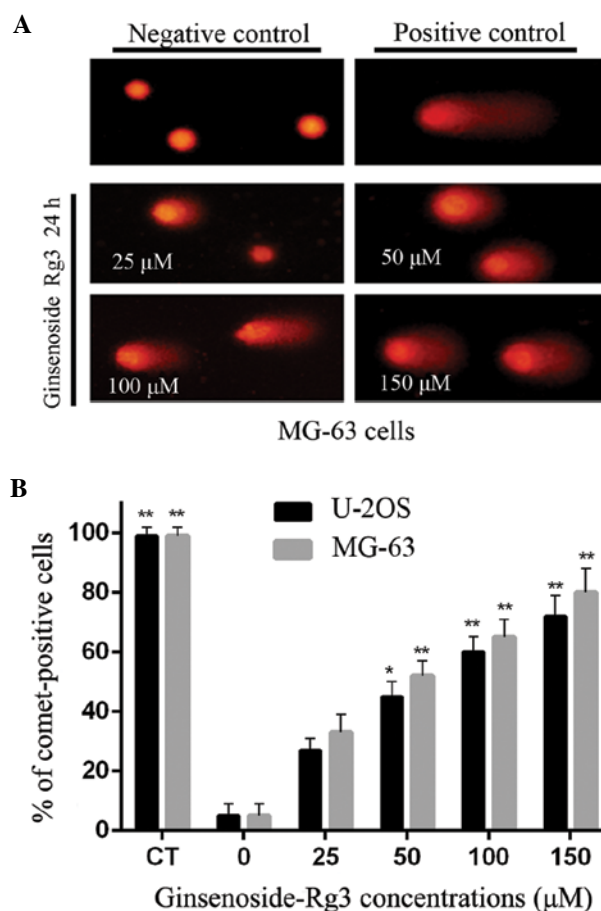


Figure 2. Comet assay evidence of DNA damage (A) as observed on alkaline gel electrophoresis with MG-63 cells (original magnification, $\times 200$) (CT, a positive control; MNNG, 20 mM). (B) The percentage of comet-positive cells in the MG-63 and U-2OS cell lines treated with ginsenoside Rg3. Data represent the mean values ($\pm$ SEM) of 3 independent experiments. * $P < 0.05$ and ** $P < 0.01$, significantly different relative to the negative control.

DNA and tail moment, are presented in Table I. These results demonstrated that cells exposed to ginsenoside Rg3 exhibited more severe DNA damage than the negative control samples. Similar trends of DNA damage induced by ginsenoside Rg3 were noted in the MG-63 and U-2OS cells.

Table I. Parameters of DNA damage in the comet assays for MG-63 and U-2OS cells that were exposed to the different concentrations of ginsenoside Rg3.

Treatment group	Cell lines/compounds	Comet assay parameters					
		Tail length (μM)		Tail DNA (%)		Tail moment	
		MG-63	U-2OS	MG-63	U-2OS	MG-63	U-2OS
Negative control	DMSO	8.15±2.58	11.45±3.42	1.54±0.42	5.12±1.71	2.15±0.69	1.12±1.02
	25 μM	16.46±6.41	21.07±1.51	8.31±1.14 ^a	16.09±3.22	9.62±2.67	6.18±0.67
	50 μM	30.01±5.21	47.42±13.52 ^b	12.42±3.52 ^b	28.66±4.16 ^b	12.13±1.64	19.33±7.47 ^b
	100 μM	37.70±7.44 ^b	56.48±8.87 ^b	19.33±1.72 ^b	35.76±4.21 ^b	19.11±2.30 ^b	23.10±3.35 ^b
	150 μM	57.24±12.47 ^b	71.06±6.70 ^b	28.25±0.46 ^b	42.31±2.99 ^b	31.11±3.24 ^b	33.26±2.36 ^b
Positive control	MNNG 20 μM	104.50±4.23 ^b	62.08±2.86 ^b	43.27±3.34 ^b	43.08±14.36 ^b	47.29±2.98 ^b	25.79±5.68 ^b

MNNG and DMSO treatments were used as the positive and negative controls, respectively. Data represent mean values (±SEM) for tail length, tail DNA (%) and tail moment of MG-63 and U-2OS cells in the alkaline comet assay. ^aP<0.05 and ^bP<0.01 represented the significance of DNA damage.

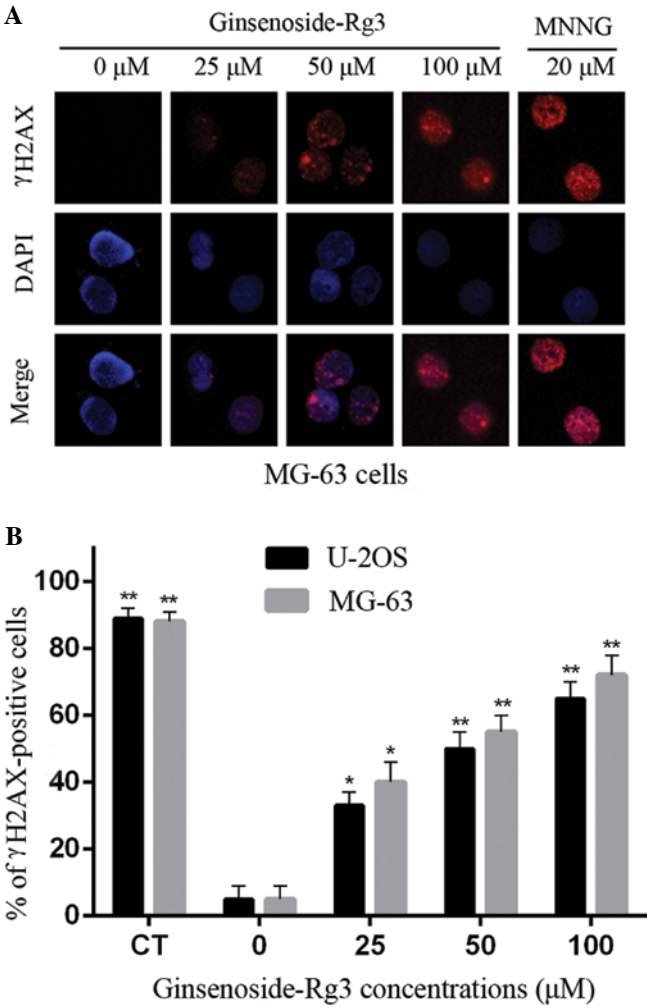


Figure 3. DNA double-strand breaks as illustrated by γ H2AX focus formation in MG-63 and U-2OS cells. (A) Representative immunofluorescent images of γ H2AX focus formation in MG-63 cells. The anti- γ H2AX monoclonal antibody was used to detect DNA damage foci immunofluorescence, and DAPI was used for nuclear staining (original magnification, $\times 400$). (B) The percentage of γ H2AX-positive (foci ≥ 4) MG-63 and U-2OS cells treated with 20 mM MNNG (CT) and 0, 25, 50 and 100 mM ginsenoside Rg3 for 24 h. Data are presented as the mean values ($\pm$ SEM) of 3 independent experiments. *P<0.05 and **P<0.01 represent significant differences from the negative control.

γ H2AX focus staining for DNA double-strand breaks. A threshold of ≥ 4 γ H2AX foci per cell was determined as optimal for quantifying the extent of DNA damage (15). Ginsenoside Rg3 caused a concentration-dependent increase in the formation of γ H2AX foci in MG-63 and U-2OS cells. Representative immunofluorescent images of the phosphorylation of histone H2AX in γ H2AX-stained MG-63 cells are shown in Fig. 3A. Negative-control MG-63 and U-2OS cells had few γ H2AX foci ($\sim 5\%$ of cells contained >4 foci). All of the treatments with ginsenoside Rg3 and MNNG induced focus formation, thereby increasing the percentage of γ H2AX-positive cells. Ginsenoside Rg3 and MNNG both exhibited distinct concentration-dependent effects (P<0.01) on γ H2AX focus formation in MG-63 and U-2OS cells (Fig. 3B).

Effects of ginsenoside Rg3 on MNNG-induced DNA damage in human fibroblasts. Ginsenoside Rg3 was reported to have

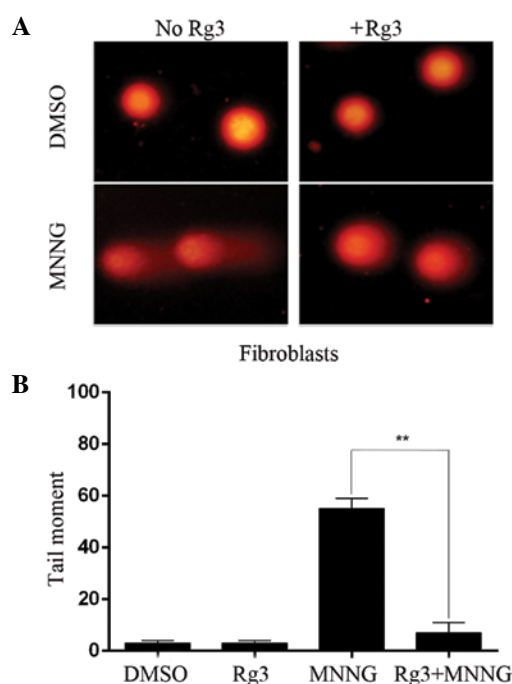


Figure 4. Protective effect of ginsenoside Rg3 on MNNG-induced DNA damage as illustrated by an alkaline comet assay in human fibroblasts. Cells were cotreated with ginsenoside Rg3 (50 mM) and MNNG (20 mM) for 24 h. (A) Representative images of comets (original magnification, x200). (B) The results represent mean values ($\pm$ SEM) of the tail moment as measured for 200 comets. **P<0.01 represents a significant difference.

a cytoprotective effect (9). We, therefore, sought to investigate the ability of ginsenoside Rg3 to protect human fibroblasts against MNNG-induced DNA damage. The number of MNNG-induced DNA single-strand breaks was measured using the alkaline comet assay. The extent of DNA damage was significantly greater in the MNNG treatment group as compared to the DMSO treatment group. In the cells pretreated with 50 mM ginsenoside Rg3 in combination with MNNG as opposed to MNNG alone, the tail moments were significantly decreased. Treatment with ginsenoside Rg3 alone did not induce any genotoxicity in the human fibroblasts (Fig. 4). The cytoprotective effect was further confirmed by staining γ H2AX foci. The percentage of γ H2AX-positive cells in the DMSO and MNNG treatment group was 5.3 and 86.9%, respectively. Ginsenoside Rg3 was effective in reducing the proportion of cells with MNNG-induced DNA strand breakage from 86.9 to 28.3% (Fig. 5).

Effects of ginsenoside Rg3 on MNNG-induced apoptosis in human fibroblasts. The rate of cell apoptosis was determined by flow cytometry. Representative graphs of cells treated with ginsenoside Rg3 and MNNG for 24 h after double staining with Annexin V-FITC and PI are shown in Fig. 6A. The rate of apoptosis in the MNNG-treated cells was $44.7 \pm 2.0\%$. When cells were treated with ginsenoside Rg3 (50 mM) prior to MNNG administration, the number of apoptotic human fibroblasts as measured 24 h after pre-treatment was significantly decreased ($12.3 \pm 2.1\%$) (Fig. 6B). Cells in the DMSO and ginsenoside Rg3 groups showed low rates of apoptosis. MNNG induced a ladder-like pattern on agarose gel (Fig. 6C).

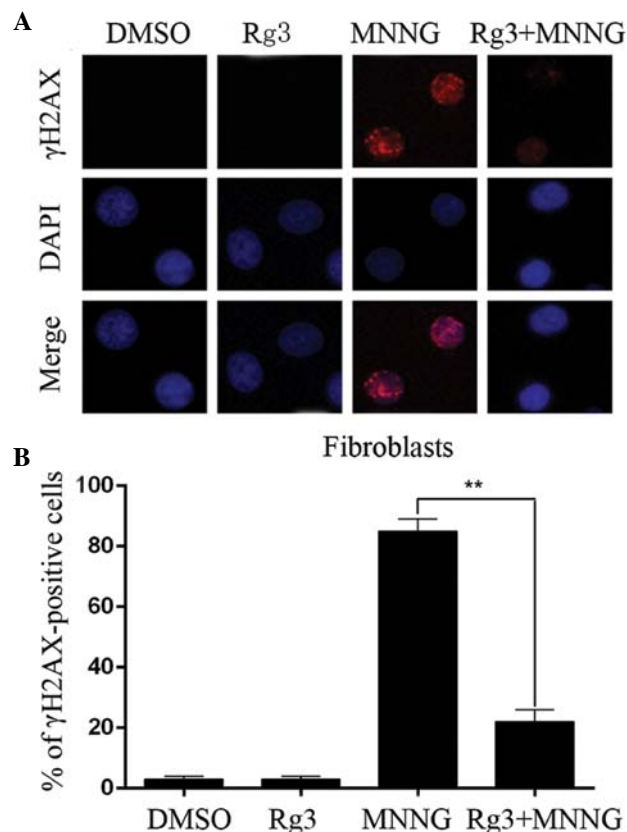


Figure 5. Protective effect of ginsenoside Rg3 on MNNG-induced double-strand breaks was illustrated by γ H2AX focus formation in human fibroblasts. Cells were cotreated with ginsenoside Rg3 (50 mM) and MNNG (20 mM) for 24 h. (A) Images of γ H2AX foci. The anti- γ H2AX monoclonal antibody was used to detect DNA damage foci immunofluorescence, and DAPI was used for nuclear staining (original magnification, x400). (B) The percentage of γ H2AX-positive (foci ≥ 4) cells. Data are presented as the mean values ($\pm$ SEM) of 3 independent experiments. **P<0.01 represents a significant difference.

Cotreatment with ginsenoside Rg3 (50 mM) induced minimal DNA fragmentation in human fibroblasts. These results suggest that ginsenoside Rg3 alleviates MNNG-induced cytotoxicity.

Discussion

Ginseng, an ancient and famous herbal drug in traditional Chinese medicine, has been used in Chinese folklore for more than 5,000 years. The most important pharmacological components in ginseng are ginsenosides. Ginsenoside Rg3 is the primary ginsenoside in ginseng. The compound can enhance immunity (16), protect against the effects of free radicals (17), suppress the invasion and metastasis of various carcinoma cells (18) and inhibit tumor angiogenesis (19).

The cytoprotective effect of ginsenosides has been demonstrated in various studies. Ginsenoside Rg3 was found to protect against DNA damage and cell apoptosis by reducing oxidative stress (10), while ginsenoside 20(S)-protopanaxatriol protected endothelial cells against oxidative stress through the regulation of intracellular redox status (20).

Osteosarcoma is the most common type of bone cancer, and the sixth most common type of cancer in children. Before any major surgery to remove the tumor is undertaken,

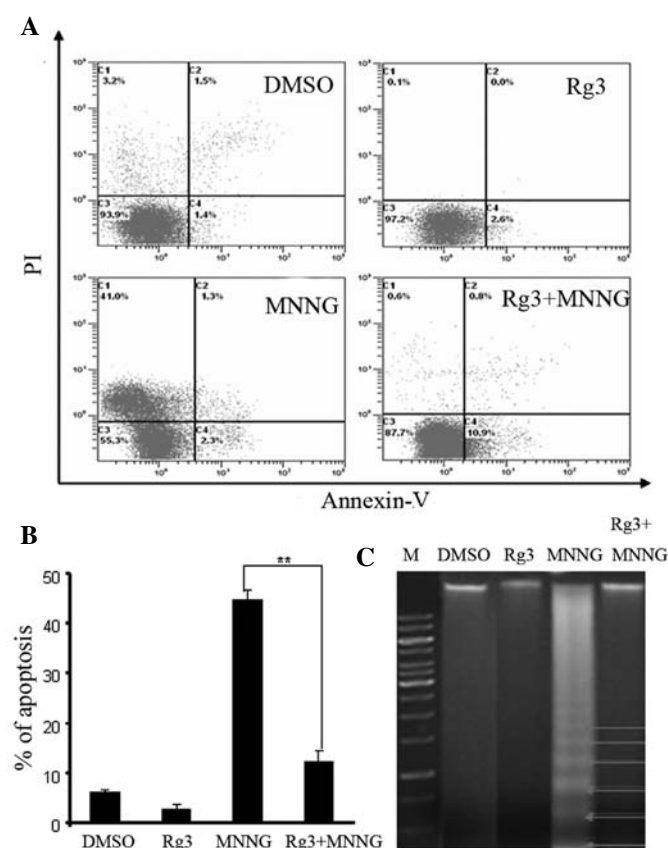


Figure 6. Protective effect of ginsenoside Rg3 on MNNG-induced apoptosis in human fibroblasts. (A) Flow cytometric analysis of apoptosis using Annexin V and PI. (B) The apoptotic rate of human fibroblasts treated with ginsenoside Rg3 (50 mM) and MNNG (20 mM) for 24 h. Data are presented as the mean values ($\pm$ SEM) of 3 independent experiments. ** $P < 0.01$ represents a significant difference. (C) DNA fragmentation on an agarose gel. DNA isolated from renal tissues of experimental rats was loaded onto 1% (w/v) agarose gels. M, marker (1-kb DNA ladder).

chemotherapy is usually administered to shrink the tumor and facilitate surgery. It may also kill any cancer cells that have spread to other areas of the body. Conventional chemotherapeutic agents such as cyclophosphamide are often toxic not only to tumor cells but also to normal cells, limiting their therapeutic use in clinics. Natural products are potentially valuable sources for the development of new anticancer drugs due to their weak side-effects (21). However, no previous report has examined whether ginsenoside Rg3 can influence human osteosarcoma cell activity or induce DNA damage. Here, we confirmed that ginsenoside Rg3 inhibits cell proliferation in human osteosarcoma cells, particularly in MG-63 and U-2OS cells.

The alkaline comet assay is a form of single-cell gel electrophoresis that can be used to detect DNA damage (22). γ H2AX focus formation can be used in this capacity as well. The alkaline comet assay is a sensitive fluorescence microscope-based method that can be used to detect DNA damage (22) caused primarily by DNA strand breaks, DNA adduct formations, and DNA-DNA and DNA-protein cross-links (23). Moreover, the formation of DNA double-strand breaks could induce the formation of γ H2AX aggregations in nuclei. γ H2AX focus formation has been suggested as a sensitive way to detect DNA double-strand breaks (24). The phosphorylation of histone

H2AX can be induced by replication stress (25), ionizing radiation (26), exogenous stress (27) and drugs that cause DNA damage (28). A threshold of ≥ 4 γ H2AX foci per cell is optimal for determining the severity of DNA damage (15). We, therefore, employed the alkaline comet assay and measurements of the formation of γ H2AX foci to detect DNA damage induced by ginsenoside Rg3 in MG-63 and U-2OS cells. Our results demonstrated a concentration-dependent increase in the size of comet tails with a concomitant reduction in head size. The images of representative comets clearly demonstrated the amount of broken DNA liberated from the heads of the comets during electrophoresis following treatment with increasing ginsenoside Rg3 concentrations. A threshold of ≥ 4 γ H2AX foci per cell was optimal for measuring the extent of DNA damage. This helped in efforts to identify the cellular DNA damage caused by ginsenoside Rg3. Treatment with ginsenoside Rg3 demonstrated an increase in the number of γ H2AX-positive cells; approximately half of these cells were positive following treatment at 25 mM, with almost 76% positive cells noted following treatment with a 100 mM solution (Fig. 3). In the present study, we demonstrated that the presence of γ H2AX foci may be indicative of DNA strand breaks, which can be confirmed by comet assay. These results are in concordance with those reported in another study, showing that ginsenoside Rg3 mediates antiproliferative and apoptotic activity in cancer cells (29).

Previous research has shown that MNNG can induce the apoptosis of fibroblasts (14). Flow cytometry and the DNA ladder assay were used to detect the ability of ginsenoside Rg3 to protect against MNNG-induced apoptosis in human fibroblasts. The ability of ginsenoside Rg3 to protect against MNNG-induced DNA damage was also evaluated using alkaline comet assay and γ H2AX focus formation. The results obtained showed that cotreatment with ginsenoside Rg3 significantly decreased MNNG-induced DNA damage and apoptosis.

These results were consistent with those of other studies. For example, red ginseng protected cells from *Helicobacter pylori*-induced DNA damage and cytotoxicity (30). Poon *et al* (9) showed that ginsenoside 20(S)-Rg3 can significantly decrease BaP-induced DNA damage using the TUNEL and comet assays in human dermal fibroblasts. Ginsenoside 20(S)-Rg3 protected against cyclophosphamide-induced DNA damage and cell apoptosis in mouse bone marrow cells and peripheral lymphocyte cells (10). Ginseng was also found to inhibit micronucleus formation and chromosomal instability (31,32).

We found that ginsenoside Rg3 protected against the effects of exposure to environmental contaminants. However, further *in vivo* studies are required to elucidate the cytoprotective effects of ginsenoside Rg3 in cells vulnerable to the genotoxic effects of environmental contaminants. Zhou and Elledge (33) indicated that organisms respond to DNA damage (DNA strand breaks) by activating a complex damage response pathway. This pathway regulates known responses such as cell cycle arrest and apoptosis. Previous reports also demonstrated that ginsenoside Rg3 treatment increases the amount of time spent by a cell in the G_2/M phase, and therefore, the likelihood that agents designed to damage DNA will in fact trigger apoptosis (34).

There are some limitations to the present study. It is not clear whether the induction of cell cycle arrest and apoptosis were directly related to genotoxic effects. Further studies will be necessary to elucidate the underlying mechanism.

In conclusion, ginsenoside Rg3 is a strong genotoxic agent that induces DNA damage in human osteosarcoma cells. Moreover, ginsenoside Rg3 protected normal human fibroblasts against the DNA damage and apoptosis induced by MNNG treatment *in vitro*. Therefore, ginsenoside Rg3 may represent a potential chemopreventive agent for the treatment of patients with osteosarcomas.

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