

# The mitochondrial pathway is involved in American ginseng-induced apoptosis of SW-480 colon cancer cells

CHONG-ZHI WANG<sup>1,2</sup>, XIAO-LI LI<sup>1,2</sup>, QIAN-FEI WANG<sup>3</sup>, SANGEETA R. MEHENDEALE<sup>1,2</sup>, ANNA B. FISHBEIN<sup>1,2</sup>, AUNG H. HAN<sup>1,2</sup>, SHI SUN<sup>1,2</sup> and CHUN-SU YUAN<sup>1,2,4</sup>

<sup>1</sup>Tang Center for Herbal Medicine Research, <sup>2</sup>Departments of Anesthesia and Critical Care, and <sup>3</sup>Medicine, <sup>4</sup>Committee on Clinical Pharmacology and Pharmacogenomics, The Pritzker School of Medicine, University of Chicago, Chicago, IL 60637, USA

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**Abstract.** Numerous effective anticancer drugs have been developed from botanical sources, and there remains a significant untapped resource in herbal medicines. In this study, we evaluated the chemical composition of extracts from American ginseng after steaming, the antiproliferative effects of the ginsenosides in the extracts on SW-480 human colorectal cancer cells, and their apoptotic mechanisms. American ginseng roots were steamed at 120°C for 2 or 4 h. Representative ginsenosides in the unsteamed and steamed extracts were determined using HPLC. The antiproliferative effects of the ginsenosides Rb1, Rg3 and Rh2 on SW-480 cells were determined by the MTS method. The effect of extract steamed for 4 h on apoptosis of SW-480 cell was assayed by flow cytometry after staining with annexin V/PI. The expression of 84 apoptotic-related genes, including TNF, mitochondria and p53 pathways, was determined using real-time quantitative PCR array analysis. The mitochondrial membrane potential ( $\Delta\psi_m$ ) was analyzed after staining with FC-1. Steaming of American ginseng increased Rg3 and Rh2 content and antiproliferative activity significantly. The quantitative PCR array data demonstrated that multiple genes in mitochondrial pathway are involved in American ginseng-induced apoptosis of SW-480 cells and the expression profiling was validated by the cellular functional assay. The mitochondrial pathway may play a key role in American ginseng-mediated cancer cell apoptosis.

## Introduction

Human colorectal cancer is a leading cause of cancer-related death in nearly all developed countries and the second most prevalent cancer worldwide. In the USA, the expected number of new colon cancer diagnoses in 2008 is 148,810 with 49,960 deaths (1). Half of all patients diagnosed with colorectal cancer eventually die from the disease. Less than 10% of patients with metastatic colorectal cancer will survive more than five years after diagnosis. Current treatment of colon cancer is surgical resection combined with chemotherapy using cytotoxic drugs and radiation therapy (2,3). Since this therapy is moderately successful for late stage cancers, preventive strategies and novel approaches for treatment of colorectal cancer are required.

Numerous effective anticancer drugs have been developed from botanical sources, and there remains a significant untapped resource in herbal medicines. Botanicals could potentially contain effective anticancer compounds for use alone or as adjuncts to existing chemotherapy to improve efficacy and reduce drug-induced toxicity (4-6).

American ginseng is an obligate shade perennial native to eastern North America. The commonly used part of the plant is the root, which is harvested in late summer to fall after several years of cultivation (7). American ginseng extract inhibited the growth of breast cancer cells (8) and enhanced the antiproliferative effect of cisplatin on human breast cancer cells, suggesting that it possesses anticancer activity (9). In separate studies, we have shown that steam-processing significantly improved the anticancer activity of ginseng on human colorectal cancer cells. The extract from roots steamed for 2 h had a greater effect than that of roots steamed for 1 h (10,11). In our studies, however, only the ginsenoside content of the crude plant material was determined, although the extract of these botanicals is often taken as a dietary supplement. Yet the mechanism whereby American ginseng root extract prevent cancer cell proliferation has not been studied.

In the present study, the content of representative ginsenosides in unsteamed and steamed extracts of American ginseng root was determined. Processing time extended up to 4 h before analysis of ginsenoside content with high

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*Correspondence to:* Dr Chun-Su Yuan, Tang Center for Herbal Medicine Research, and Department of Anesthesia and Critical Care, Pritzker School of Medicine, University of Chicago, 5841 S. Maryland Avenue, MC 4028, Chicago, IL 60637, USA  
E-mail: cyuan@dacc.uchicago.edu

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performance liquid chromatography (HPLC). Subsequently, the effects of the steamed extract on apoptosis of SW-480 colorectal cancer cells were assayed by flow cytometry, and the expression level of key genes involved in apoptotic pathways was determined using real-time quantitative PCR array.

## Materials and methods

**Reagents.** Ginsenoside standards Rb1, Rc, Rd, Re, Rg2, Rg3 and Rh2, purchased from Delta Information Center for Natural Organic Compounds (Xuancheng, AH, P.R. China), were of biochemical-reagent grade and at least 95% pure as confirmed by HPLC. HPLC grade ethanol, n-butanol, acetonitrile and absolute ethanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Trypsin, Leibovitz's L-15 medium, fetal bovine serum (FBS), and penicillin/streptomycin solution (x200) were obtained from Mediatech, Inc. (Herndon, VA, USA). A CellTiter 96 Aqueous One Solution cell proliferation assay kit was obtained from Promega (Madison, WI, USA). Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were obtained from BD Biosciences (San Diego, CA, USA).

**Plant materials.** The root of *Panax quinquefolius* L. was collected from Roland Ginseng Limited Liability Company (Wausau, WI, USA). The plant material was identified by Dr Chong-Zhi Wang according to the United States Pharmacopoeia NF 21, monograph: American ginseng (*Panax quinquefolius* L.). The voucher specimen was deposited at the Tang Center for Herbal Medicine Research at the University of Chicago.

**Steaming treatment and extraction.** The roots of American ginseng were steamed at 120°C for 2 or 4 h. The fresh and steamed roots were lyophilized to obtain dried samples. The extraction process was as follows: The dried roots were ground and extracted with 70% ethanol. The solvent of the extract solution was evaporated under vacuum. The dried extract was dissolved in water and then extracted with water-saturated n-butanol. The n-butanol phase was evaporated under vacuum and then lyophilized.

**HPLC analysis.** HPLC analysis was conducted on a Waters 2960 instrument with a Waters 996 photodiode array detector (Milford, MA, USA). The separation was carried out on an Alltech Ultrasphere C18 column (5  $\mu$ , 250x3.2 mm I.D.) (Deerfield, IL, USA) with a guard column (Alltech Ultrasphere C18, 5  $\mu$ , 7.5x 3.2 mm I.D.). Acetonitrile (solvent A) and water (solvent B) were used. Gradient elution started with 18% solvent A and 82% solvent B, and was changed to 21% A for 20 min, to 26% A for 3 min, and held for 19 min; then was changed to 36% A for 13 min, to 50% A for 9 min, to 95% A for 2 min and held for 3 min; and finally changed to 18% A for 3 min and held for 8 min. The flow rate was 1.0 ml/min and the detection wavelength was set to 202 nm. All the tested solutions were filtered through Millex 0.2- $\mu$ m nylon membrane syringe filters (Millipore Co., Bedford, MA, USA) before use. The contents of ginsenosides in each sample were calculated using standard curves of ginsenosides.

**Cell line and culture.** The human colorectal cancer cell line SW-480 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in Leibovitz's L-15 medium supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere without CO<sub>2</sub> at 37°C.

**Cell proliferation analysis.** Unsteamed and steamed American ginseng root extracts and ginsenosides were dissolved in 50% ethanol and were stored in 4°C before use. The SW-480 cells were seeded in 96-well plates. After 1 day, various concentrations of extracts/ginsenosides were added to the wells. The final concentration of ethanol was 0.5%. The controls were exposed to culture medium containing 0.5% ethanol without drugs. All the experiments were performed in triplicate and repeated 3 times. The cell proliferation was evaluated using an MTS assay according to the manufacturer's instructions. Briefly, at the end of the drug exposure period, the medium was replaced with 100  $\mu$ l of fresh medium. 20  $\mu$ l of MTS reagent (CellTiter 96 aqueous solution) was added to each well, and the plate was returned to the incubator for 1-2 h. A 60- $\mu$ l aliquot of medium from each well was transferred to an ELISA 96-well plate and its absorbance at 490 nm was recorded. The results were expressed as percentage of the control (ethanol controls set at 100%).

**Apoptosis assay.** The SW-480 cells were seeded in 24-well tissue culture plates. After culturing for 1 day, the medium was changed and extract from American ginseng root steamed for 4 h was added. After treatment for 48 h, the cells floating in the medium were collected. The adherent cells were detached with 0.05% trypsin. Then the culture medium containing 10% FBS (and floating cells) was added to inactivate the trypsin. After being pipetted gently, the cells were centrifuged for 5 min at 1500 x g. The supernatant was removed and the cells were stained with annexin V-FITC or annexin V-FITC/propidium iodide (PI). The cells were analyzed immediately after staining using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and FlowJo 7.1.0 software (Tree Star, Ashland, OR, USA). For each measurement, at least 20,000 cells were counted.

**Reverse transcription and real-time quantitative PCR array analysis.** The expression of apoptotic genes was analyzed by reverse transcription-PCR (RT-PCR; Applied Biosystems 7300, Foster City, CA) using an RT<sup>2</sup> real-time SYBR Green/ROX gene expression assay kit (SuperArray Bioscience Corp., Frederick, MD). Total RNA was isolated from solvent control and treated SW-480 cells using the RNAeasy kit (Qiagen, Hilden, Germany). The first strand of cDNA was synthesized from 2  $\mu$ g total RNA using a SuperScript II First-strand synthesis system (Invitrogen). Gene profiling was performed as described by the manufacturer using the RT<sup>2</sup>-profiler PCR array of the human Apoptosis Signaling Pathway including 84 apoptotic-related genes (catalog number: PAHS-012, SuperArray Bioscience). Briefly, quantitative real-time RT-PCR was performed in a reaction volume of 25  $\mu$ l including 1  $\mu$ l cDNA. PCR conditions were as follows: 95°C for 15 min followed by 40 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. GAPDH was

**SPANDIDOS** an internal reference gene to normalize the  
**PUBLICATIONS** n of apoptotic genes. Relative quantification of

apoptotic genes was analyzed by the comparative threshold cycle (Ct) method. A Ct cycle was used to determine the expression level in control and in SW-480 cells treated with American ginseng extract steamed for 4 h. Thus, expression levels were expressed as an n-fold difference relative to the calibrator. For each sample, the apoptotic gene Ct value was normalized using the formula:  $\Delta Ct = Ct(\text{apoptotic genes}) - Ct(\text{GAPDH})$ . To determine relative expression levels, the following formula was used:  $\Delta\Delta Ct = \Delta Ct(\text{Treated}) - \Delta Ct(\text{Control})$ . The value was used to plot the expression of apoptotic genes using the expression  $2^{-\Delta\Delta Ct}$ .

**Mitochondrial membrane potential ( $\Delta\psi_m$ ) analysis.**  $\Delta\psi_m$  was estimated using JC-1 (Molecular Probes, Eugene, OR, USA). This fluorescent compound exists as a monomer at low concentrations. At higher concentrations, it forms aggregates. Fluorescence of the JC-1 monomer is green. That of the aggregate is red. Mitochondria with intact membrane potential concentrate JC-1 into aggregates, which fluoresce red. De-energized mitochondria cannot concentrate JC-1 and fluoresce green (12). SW-480 cells were treated for 24 h with steamed extract at 100, 150 or 200  $\mu\text{g/ml}$ . Control cells were grown in medium containing the same amount of ethanol as treated cells. Then, the adherent cells were incubated in 0.5 ml of medium containing JC-1 (2.5  $\mu\text{g/ml}$ ) for 20 min at 37°C, and images were taken with a Nikon microscope ECLIPSE E800 (Nikon Corporation, Champigny-sur-Marne, France).

**Statistical analysis.** The data are presented as mean  $\pm$  standard deviation (SD) with  $n=3$ . A one-way ANOVA determined whether the results had statistical significance. In some cases, the Student's t-test was used for comparing two groups. The level of statistical significance was set at  $P<0.05$ .

## Results

**Effect of steaming treatment on the ginsenoside composition.** It is believed that the bioactive compounds in American ginseng are saponins, commonly referred to as ginsenosides (13). In our previous studies (10,11), after steaming treatment of American ginseng root, the contents of ginsenosides Rb1, Rc, Rd and Re decreased, while the content of ginsenoside Rg3 increased. Ginsenosides Rb1, Rc and Rd belong to the protopanaxadiol group. The effect of steaming on the protopanaxadiol group ginsenosides is shown in Fig. 1. During steaming, the protopanaxatriol group ginsenosides Re and Rg1 changed to Rg2 and Rh1. Since the ginsenoside content of the protopanaxatriol group of American ginseng is relatively low, we did not show their structures. In this study, we assayed the ginsenoside composition in the extracts.

From the HPLC chromatograms of extracts from unsteamed and steamed American ginseng roots, ginsenosides Rb1 and Re were the major constituents in unsteamed extract (Fig. 2A). After 4 h of steaming, ginsenosides Rg2 and Rg3 became the main constituents (Fig. 2B). During heat-processing, the peak areas of ginsenosides Re, Rb1, Rc and Rd decreased significantly, while ginsenosides Rg2 and Rg3 increased (Fig. 2A and B). The changes of constituents

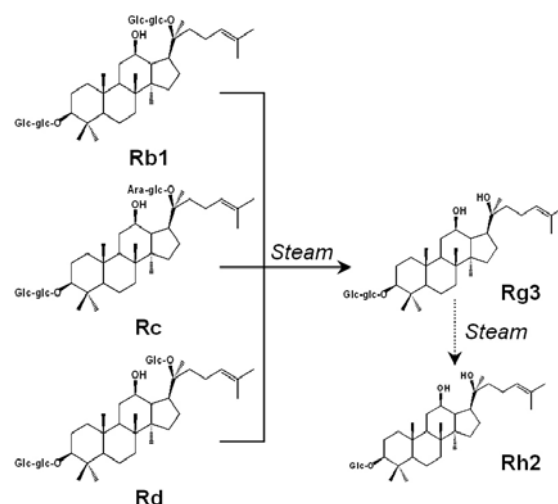


Figure 1. Transformation of ginsenosides in American ginseng root during steaming treatment.

in the extracts are shown in Fig. 2C. We found the following percentages of ginsenosides in unsteamed extract: Re 19.0%; Rb1 34.2%; Rc 3.4% and Rd 6.5%. After 4 h of steaming, the contents were decreased to undetected, 1.6, 0.1 and 0.9%, respectively. In unsteamed extract, Rg2 was 0%; Rg3 0.06% and Rh2 0.06%. In extract steamed for 2 h, Rg2 was 3.2%; Rg3 5.9% and Rh2 0.3%. After 4 h of steaming, values changed to 2.3, 7.8 and 1.2%, respectively. Steaming for 4 h increased the content of Rg3 and Rh2 significantly (Fig. 2C).

**Effects of extracts and ginsenosides on SW-480 human colorectal cancer cells.** Using human colorectal cancer cell line SW-480, the antiproliferative effect of unsteamed and steamed American ginseng root extracts was evaluated. At 150  $\mu\text{g/ml}$ , unsteamed extract inhibited cell growth by 6.4%. Steamed for 2 h, the extract inhibited cell growth by 13.5%, and steamed for 4 h, the extract inhibited cell growth by 82.4% ( $P<0.01$  vs. control). At 250  $\mu\text{g/ml}$ , unsteamed extract inhibited cell growth by 13.5%. And steamed for 2 h, the extract inhibited cell growth by 77.6% ( $P<0.01$  vs. control). Steamed for 4 h, the extract inhibited cell growth absolutely (Fig. 3A). These results show the antiproliferative effects of the extract on human colorectal cancer cells. Steaming for 4 h made the extract more potent than steaming for 2 h.

Three representative ginsenosides were used to test their antiproliferative effects on SW-480 colorectal cancer cells. Among them, ginsenoside Rb1 was a major constituent in unsteamed American ginseng roots. Two other ginsenosides, Rg3 and Rh2, were the main constituents in steamed roots. After 72 h of treatment at 10-100  $\mu\text{M}$ , ginsenoside Rb1 did not show antiproliferative effects on SW-480 cells. At 60  $\mu\text{M}$ , ginsenoside Rg3 inhibited cell growth by 4.3%, and Rh2 inhibited cell growth by 99.3% ( $P<0.01$  vs. untreated control). At 100  $\mu\text{M}$ , ginsenosides Rg3 and Rh2 inhibited cell growth by 31.5 and 98.8%, respectively (both  $P<0.01$  vs. untreated control, Fig. 3B). Ginsenosides Rg3 and Rh2 showed a positive antiproliferative effect on SW-480 cells, and Rh2 showed a stronger antiproliferative effect than Rg3.

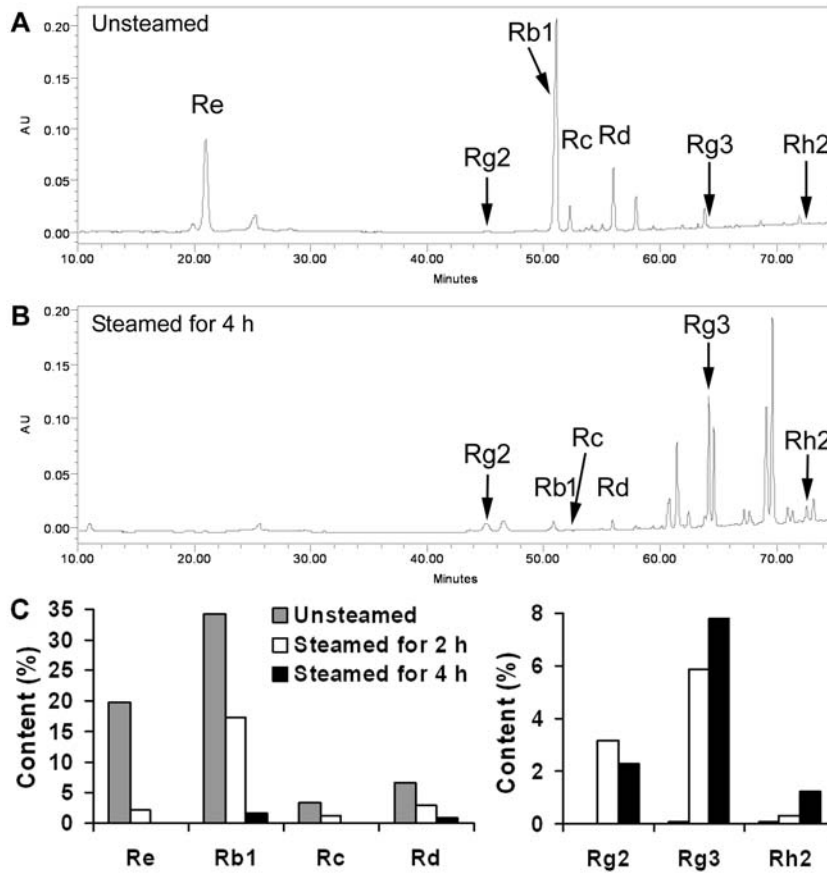


Figure 2. HPLC analysis of American ginseng extracts. Chromatograms of extracts from unsteamed American ginseng (A), and American ginseng steamed for 4 h (B). Percentages of ginsenosides in extracts are shown in (C) left and right.

To evaluate the contribution of Rg3 and Rh2 to the antiproliferative effects of extract steamed for 4 h, we compared the effects among the extract and ginsenosides. At 200  $\mu\text{g/ml}$ , the extract inhibits cell growth absolutely (Fig. 3A). At 200  $\mu\text{g/ml}$  the extract contains 19.9  $\mu\text{M}$  of Rg3 and 3.9  $\mu\text{M}$  of Rh2. Yet with 30  $\mu\text{M}$  of Rg3 or 10  $\mu\text{M}$  of Rh2, no antiproliferative effects were observed (Fig. 3B), suggesting that other potent antiproliferative compound(s) exist in extract steamed for 4 h.

*Induction of apoptosis of SW-480 cells.* To explore the potential mechanism through which American ginseng extract inhibits cell growth, cell apoptosis was assayed by flow cytometry after staining with annexin V. As shown in Fig. 4A, after treatment with extract steamed for 4 h, annexin V positive cells were observed, and the percentage of apoptotic cells was increased markedly. Compared to control (9.5%), after treatment with 100, 150 or 200  $\mu\text{g/ml}$  of the extract, the percentage of apoptotic cells was increased to 15.8, 31.9 and 55.3%, respectively.

To further characterize the observed apoptotic phenotype, we carried out double staining of annexin V and PI in SW-480 cells treated with extract (Fig. 4B). Annexin V can be detected in both the early and late stages of apoptosis. PI enters the cell in late apoptosis or necrosis. Viable cells were negative for both annexin V and PI (lower left quadrant); early apoptotic cells were positive for annexin V and negative for PI (lower right quadrant); late apoptotic or necrotic cells

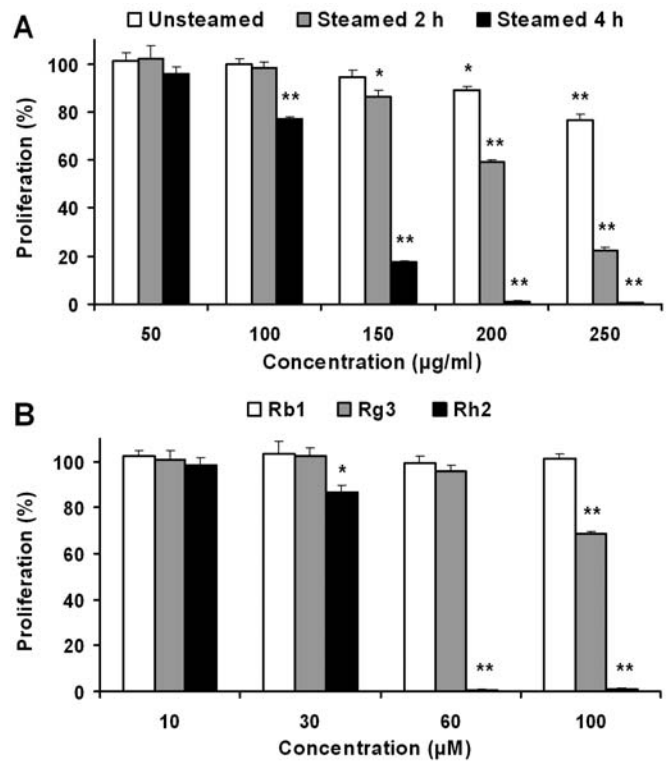


Figure 3. Effects of American ginseng extracts and ginsenosides on proliferation of SW-480 human colorectal cancer cells. Cells were treated with extracts (A) or ginsenosides (B) for 72 h, and then assayed by the MTS method. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control.



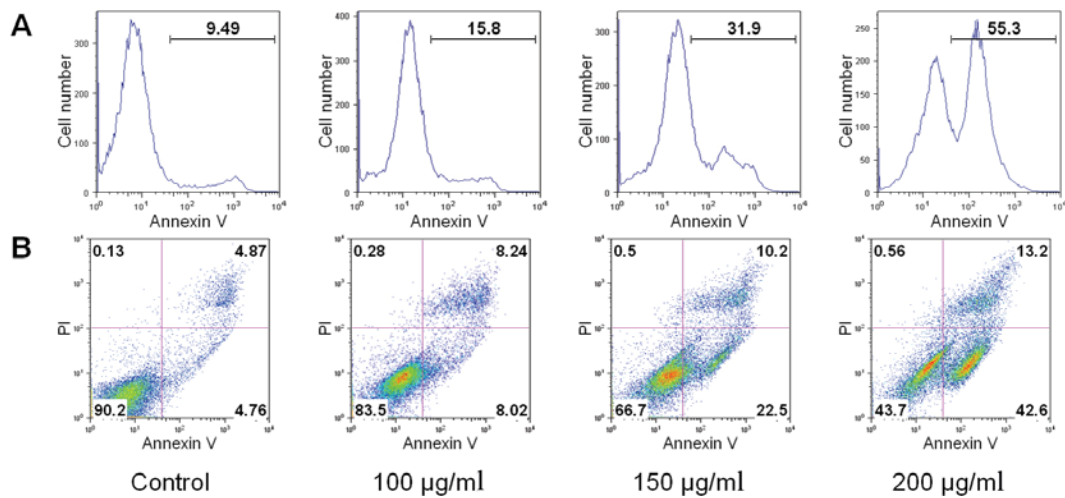


Figure 4. Apoptosis assay using flow cytometry after staining with annexin V-FITC (A) or annexin V-FITC and PI (B). SW-480 cells were treated for 48 h with 100, 150 or 200 µg/ml of extract steamed for 4 h. Early apoptotic cells are in lower right quadrant (B).

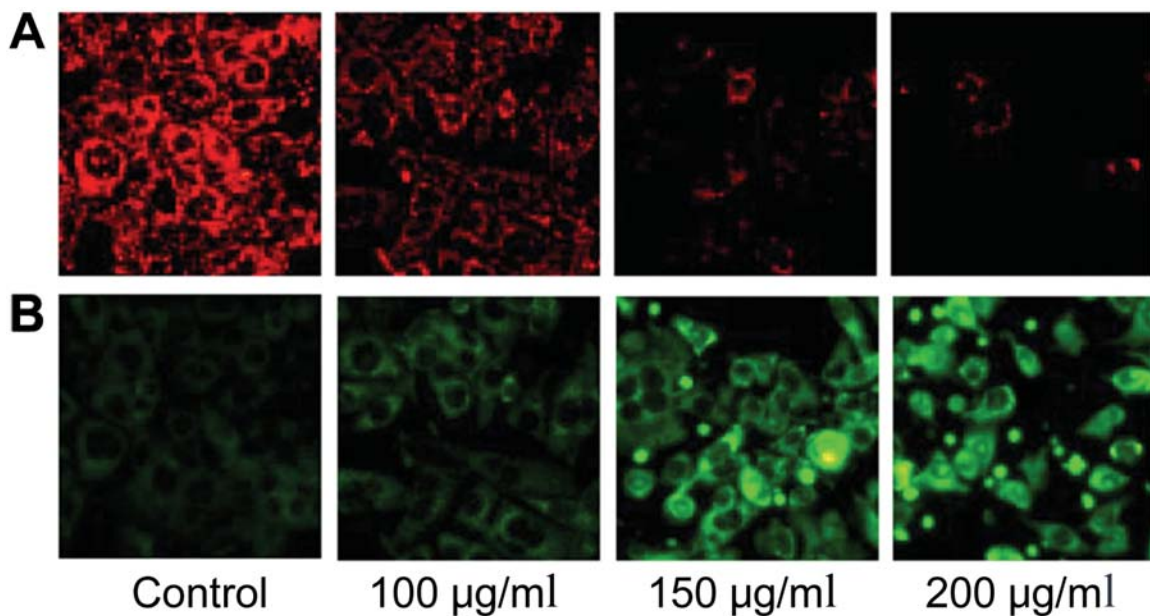


Figure 5. Analysis of the mitochondrial membrane potential ( $\Delta\psi_m$ ) after treatment. The SW-480 cells were treated for 24 h with American ginseng root extract steamed for 4 h and then stained with 2.5 µM of JC-1 for 20 min. Red fluorescence (A) represents mitochondria with intact membrane potential. Green fluorescence (B) represents de-energized mitochondria. Images were taken with a fluorescence microscope.

displayed both positive annexin V and PI (upper right quadrant); non-viable cells which underwent necrosis were positive for PI and negative for annexin V (upper left quadrant). After treatment for 48 h, the percentage of early apoptotic cells induced by 100, 150 or 200 µg/ml of steamed extract was 8.0, 22.5 and 42.6%, respectively. Control was 4.8% (Fig. 4B). Steamed American ginseng root extract induced apoptosis in SW-480 cells significantly. The anti-proliferative effect of American ginseng extract steamed for 4 h was mediated by the induction of apoptosis.

*Effects of steamed extract on apoptotic pathways of SW-480 cells.* To identify specific pathway(s) that mediate apoptosis induced by American ginseng extract, we focused on 84 apoptotic-related genes. The expression level was determined with qPCR for the set of genes that are involved in TNF,

mitochondria and p53 pathways. Extract steamed for 4 h inhibited 63 genes and induced 21 genes. Gene expression changes two-fold or greater vs. untreated control are listed in Table I. Steamed extract increased the transcripts of caspase 5 (CASP5),  $\alpha$ -growth arrest and DNA-damage-inducible (GADD45A), Harakin, BCL2 interaction protein (HRK), and tumor necrosis factor receptor superfamily member 9 (TNFRSF9). Steamed extract decreased the expression of B-cell CCL/lymphoma 2 (BCL2), BCL2-associated transcription factor 1 (BCLAF1), insulin-like growth factor 1 receptor (IGF1R), CD27 molecule (CD27), tumor necrosis factor (ligand) super family, member 10 (TNFSF10), and tumor protein p73 (TP73).

As shown in Fig. 4, steamed extract treatment significantly increased apoptosis in colorectal cancer cells. Apoptosis is the result of death receptor-dependent (extrinsic)

Table I. Apoptotic genes regulated by steamed extract.<sup>a</sup>

Gene symbol	Description	Fold changes vs. control
Up-regulated		
CASP5	Caspase 5, apoptosis-related cysteine peptidase	2.50
GADD45A	Growth arrest and DNA-damage-inducible, $\alpha$	3.20
HRK	Harakin, BCL2 interaction protein (contains only BH3 domain)	3.76
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	2.14
Down-regulated		
BCL2	B-cell CCL/lymphoma 2	-2.89
BCLAF1	BCL2-associated transcription factor 1	-2.51
IGF1R	Insulin-like growth factor 1 receptor	-2.17
CD27	CD27 molecule	-3.56
TNFSF10	Tumor necrosis factor (ligand) super family, member 10	-4.63
TP73	Tumor protein p73	-2.43

<sup>a</sup>RNA was extracted from SW-480 cells which were treated for 24 h with 150  $\mu\text{g/ml}$  of American ginseng extract that had been steamed for 4 h.

or independent (intrinsic or mitochondrial) mechanisms (14). The best-characterized death receptor is the tumor necrosis factor (TNF) superfamily. The corresponding ligands of the TNF superfamily comprise death receptor ligands. Steamed extract increased TNFRSF9, a TNF receptor that contributes to the clonal expansion, survival and proliferation in monocytes. Steamed extract inhibited another protein, TNFSF10, which preferentially induces apoptosis in transformed and tumor cells (15,16), suggesting that apoptosis is determined by cellular context and interactions between multiple regulatory factors. In our study the death receptor-dependent mechanism may not contribute to apoptotic induction.

HRK, BCL2 and BCLAF1 are Bcl-2 family genes that may contribute to the apoptotic induction of cancer cells through the mitochondrial pathway. The Bcl-2 family consists of pro-apoptotic (or cell death) and anti-apoptotic (or cell survival) genes. It is the balance in expression between these gene lineages that may determine the death or survival of a cell. BCL2 is an anti-apoptotic gene. HRK and BCLAF1 are two pro-apoptotic genes (17). As illustrated in Table I, steamed extract increased the expression of HRK. HRK activates Harakin which interacts with death-repressor proteins Bcl-2 (18). Steamed extract decreased the expression of BCL2 markedly. Forced BCL2 down-regulation results in mitochondrial dysfunction and induces apoptosis (18). BCLAF1, was depressed by steamed extract, demonstrating that apoptosis is induced at least partly through a mitochondrial mechanism.

*Effects of steamed extract on the mitochondrial membrane potential of SW-480 cells.* To validate the qPCR data, we performed a cellular functional assay. The spatial variation in mitochondrial membrane potential ( $\Delta\psi\text{m}$ ) was estimated using the JC-1 probe. This probe accumulates in the mitochondria because of membrane potentials. Organelles

with low  $\Delta\psi\text{m}$  accumulate a low number of JC-1 molecules and fluoresce green (485 excitation/535 emission). At high concentrations (high  $\Delta\psi\text{m}$ ), the probe aggregates exhibit a red-shifted fluorescence (535 excitation/590 emission). Loss of membrane potential is followed by a red to green shift. As illustrated in Fig. 5, untreated SW-480 cells showed red fluorescence. After treatment with 100  $\mu\text{g/ml}$  of steamed extract,  $\Delta\psi\text{m}$  decreased. After treatment with 150 or 200  $\mu\text{g/ml}$  of extract, fluorescence shifted from red to green, indicating loss of mitochondrial function. This result suggests that steamed American ginseng extract induced apoptosis by the mitochondrial pathway.

## Discussion

Botanical extracts are a complicated mixture of bioactive compounds. The concentrations of these compounds vary significantly depending on genetics, season, geographical distribution, plant growth, and production and extract processes (19,20). Therefore, botanical identification and analysis are important in the quality assurance of botanical products (21,22). In this study, we used American ginseng from Roland Ginseng, LLC because American ginseng from Wisconsin is a reliable ginseng source (23). The content of the major ginsenosides in American ginseng extracts was determined using HPLC to standardize our extracts.

Steaming American ginseng changes the constituent profiles. Previously we have reported the effect of steaming temperatures and times on ginsenoside content in the crude herb (11). In this study, the content of the main ginsenosides in the extracts was evaluated. Ginsenosides Rg3 and Rh2, two previously recognized anticancer compounds (24), were detected in unsteamed and steamed extracts. In the unsteamed extract, Rg3 and Rh2 were only trace saponins (both 0.06%). After steaming for 4 h, Rg3 and Rh2 increased to 7.8% and



SPANDIDOS PUBLICATIONS: respectively, becoming the main constituents in the (Fig. 2). *In vitro* pharmacological studies showed that

steaming American ginseng increases the antiproliferative effect significantly (Fig. 3). The antiproliferative effects of Rg3 and Rh2 may work with other compound(s) that possess greater antiproliferative effects than Rg3 and Rh2. We thought it worth evaluating the cancer cell inhibitory mechanisms of other unidentified potent compound(s) in steamed extract, even though the anticancer mechanisms of Rg3 and Rh2 have already been evaluated (25,26).

Apoptosis is considered an important mechanism in the inhibition of cancer cells of many anticancer agents (27,28). In this study, we assayed the induction of apoptosis by American ginseng extract. Extract steamed for 4 h showed potent apoptotic induction activities on SW-480 cells (Fig. 4).

To explore the apoptotic induction mechanism of steamed extract, we performed expression profiling analyses using an RT<sup>2</sup>-profiler PCR array containing 84 apoptotic-related genes. Through identification of top up- or down-regulated genes, we found that steamed extract induced SW-480 cell apoptosis through the mitochondrial pathway. This result was further confirmed by a cellular function assay of mitochondrial membrane potential. The mitochondrial pathway may contribute to apoptosis in SW-480 cells induced by steamed American ginseng. Mitochondria integrate transduction of cellular apoptotic signals and amplify the apoptotic response (12). Disruption of mitochondrial electron transport and energy metabolism is recognized as an early event in apoptosis and precedes the appearance of morphologic changes characteristic of apoptosis (18).

In addition to genes that involve the mitochondrial pathway, the RT<sup>2</sup>-profiler PCR array also includes TNF and p53 pathways. Since the cell line SW-480 is a p53 mutation, the p53 pathway does not contribute to the apoptosis (29). We found no evidence that the death receptor-dependent mechanism contributed to apoptosis induced by steamed extract. Interestingly, steamed American ginseng extract not only increased other pro-apoptotic gene expression such as that of CASP5, but also decreased the anti-apoptotic gene expression such of IGF1R. Further confirmation is needed to correlate the observed changes at the mRNA level with protein expression.

In conclusion, expression profiling on selected pathways revealed various apoptotic related genes that inhibited growth in SW-480 human colorectal cancer cells by American ginseng. The mitochondrial apoptotic pathway may play a key role in cancer chemoprevention by steamed American ginseng extract. Our expression analysis may lead to the identification of markers that predict the responsiveness of colorectal cancer cells to American ginseng treatment.

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## References

- Jemal A, Siegel R, Ward E, *et al*: Cancer statistics, 2008. *CA Cancer J Clin* 58: 71-96, 2008.
- Oehler C and Ciernik IF: Radiation therapy and combined modality treatment of gastrointestinal carcinomas. *Cancer Treat Rev* 32: 119-138, 2006.
- Matsui T, Omura K, Kawakami K, Morita S and Sakamoto J: Genotype of thymidylate synthase likely to affect efficacy of adjuvant 5-FU based chemotherapy in colon cancer. *Oncol Rep* 16: 1111-1115, 2006.
- Vickers A: Botanical medicines for the treatment of cancer: rationale, overview of current data, and methodological considerations for phase I and II trials. *Cancer Invest* 20: 1069-1079, 2002.
- Lee TI, Chen HH and Yeh ML: Effects of chan-chuang qigong on improving symptom and psychological distress in chemotherapy patients. *Am J Chin Med* 34: 37-46, 2006.
- Wu H, Yang F, Cui S, Qin Y, Liu J and Zhang Y: Hematopoietic effect of fractions from the enzyme-digested colla corii asini on mice with 5-fluorouracil induced anemia. *Am J Chin Med* 35: 853-866, 2007.
- Wang CZ, Mehendale SR and Yuan CS: Commonly used antioxidant botanicals: active constituents and their potential role in cardiovascular illness. *Am J Chin Med* 35: 543-558, 2007.
- Mehendale S, Aung H, Wang A, *et al*: American ginseng berry extract and ginsenoside Re attenuate cisplatin-induced kaolin intake in rats. *Cancer Chemother Pharmacol* 56: 63-69, 2005.
- Aung HH, Mehendale SR, Wang CZ, Xie JT, McEntee E and Yuan CS: Cisplatin's tumoricidal effect on human breast carcinoma MCF-7 cells was not attenuated by American ginseng. *Cancer Chemother Pharmacol* 59: 369-374, 2007.
- Wang CZ, Zhang B, Song WX, *et al*: Steamed American ginseng berry: ginsenoside analyses and anticancer activities. *J Agric Food Chem* 54: 9936-9942, 2006.
- Wang CZ, Aung HH, Ni M, *et al*: Red American ginseng: ginsenoside constituents and antiproliferative activities of heat-processed *Panax quinquefolius* roots. *Planta Med* 73: 669-674, 2007.
- Keshavan P, Schwemberger SJ, Smith DL, Babcock GF and Zucker SD: Unconjugated bilirubin induces apoptosis in colon cancer cells by triggering mitochondrial depolarization. *Int J Cancer* 112: 433-445, 2004.
- Wang CZ, Wu JA, McEntee E and Yuan CS: Saponins composition in American ginseng leaf and berry assayed by high-performance liquid chromatography. *J Agric Food Chem* 54: 2261-2266, 2006.
- Fulda S and Debatin KM: Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene* 25: 4798-4811, 2006.
- Cretney E, Takeda K and Smyth MJ: Cancer: novel therapeutic strategies that exploit the TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway. *Int J Biochem Cell Biol* 39: 280-286, 2007.
- Yanase N, Kanetaka Y and Mizuguchi J: Interferon-alpha-induced apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-dependent and -independent manner. *Oncol Rep* 18: 1031-1038, 2007.
- Majors BS, Betenbaugh MJ and Chiang GG: Links between metabolism and apoptosis in mammalian cells: applications for anti-apoptosis engineering. *Metab Eng* 9: 317-326, 2007.
- Willis SN and Adams JM: Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 17: 617-625, 2005.
- Ernst E: The risk-benefit profile of commonly used herbal therapies: Ginkgo, St. John's Wort, Ginseng, Echinacea, Saw Palmetto, and Kava. *Ann Intern Med* 136: 42-53, 2002.
- Ang-Lee MK, Moss J and Yuan CS: Herbal medicines and perioperative care. *JAMA* 286: 208-216, 2001.
- Wang CZ, Li P, Ding JY, Peng X and Yuan CS: Simultaneous identification of *Bulbus Fritillariae cirrhosae* using PCR-RFLP analysis. *Phytomedicine* 14: 628-632, 2007.
- Wang CZ, Li P, Ding JY, Fishbein A and Yuan CS: Discrimination of *Lonicera japonica* THUNB from different geographical origins using restriction fragment length polymorphism analysis. *Biol Pharm Bull* 30: 779-782, 2007.
- Assinewe VA, Baum BR, Gagnon D and Arnason JT: Phytochemistry of wild populations of *Panax quinquefolius* L. (North American ginseng). *J Agric Food Chem* 51: 4549-4553, 2003.
- Helms S: Cancer prevention and therapeutics: *Panax ginseng*. *Altern Med Rev* 9: 259-274, 2004.

25. Popovich DG and Kitts DD: Mechanistic studies on proto-panaxadiol, Rh2, and ginseng (*Panax quinquefolius*) extract induced cytotoxicity in intestinal Caco-2 cells. *J Biochem Mol Toxicol* 18: 143-149, 2004.
26. Min JK, Kim JH, Cho YL, *et al.*: 20(S)-Ginsenoside Rg3 prevents endothelial cell apoptosis via inhibition of a mitochondrial caspase pathway. *Biochem Biophys Res Commun* 349: 987-994, 2006.
27. Reed JC and Pellecchia M: Apoptosis-based therapies for hematologic malignancies. *Blood* 106: 408-418, 2005.
28. Wu WY, Guo HZ, Qu GQ, Han J and Guo DA: Mechanisms of pseudolaric Acid B-induced apoptosis in bel-7402 cell lines. *Am J Chin Med* 34: 887-899, 2006.
29. Huang L and Pardee AB: beta-lapachone induces cell cycle arrest and apoptosis in human colon cancer cells. *Mol Med* 5: 711-720, 1999.