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Original Article

Anticancer effects of black ginseng extract on human gastric cancer in a xenograft mouse model

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Abstract: Objective: This study investigated the potential of black ginseng extract (BGE) to prevent and treat gastric cancer by evaluating its effects on the proliferation of human gastric cancer cells and the growth of human gastric tumors implanted in mice. Materials and methods: The antitumor effects of BGE on gastric cancer were evaluated by assessing its cytotoxicity and apoptotic activity in AGS and KATO III gastric cancer cells. Additionally, in a nude mouse model with AGS cell xenografts, tumor volume, weight, size, and body weight were analyzed following oral administration of BGE, red ginseng extract (RGE), or no treatment. Subsequently, the liver and spleen were weighed, and histopathological analysis was performed to assess the non-toxicity of BGE to non-cancerous cells. Results: *In vitro* studies revealed that BGE had significant cytotoxic and apoptotic effects on AGS and KATO III gastric cancer cells. *In vivo*, oral administration of RGE and BGE to nude mice with AGS cell xenografts resulted in no increase in tumor volume or body weight, while both tumor weight and size were significantly reduced. Notably, the group treated with BGE had significantly smaller tumors and weight than the RGE-treated group. Furthermore, following oral BGE, no significant differences were observed in the weights of the liver and spleen, and no histopathological abnormalities were detected in the organs of the AGS cell xenograft mice. Conclusions: BGE exhibited anticancer activity against gastric cancer cells in vitro and in vivo, with no toxicity observed in the major organs.

Keywords: Anticancer effect, black ginseng extract, gastric cancer cell, xenograft mouse model, toxicity

Introduction

Gastric cancer is the fifth most common malignancy worldwide, with the fourth highest mortality rate among all cancers [1]. Every year, more than one million new cases are diagnosed and about 769,000 people die from the disease [2]. Standard treatments for gastric cancer include gastrectomy, chemotherapy, and radiation therapy [3]. However, these treatments often encounter significant obstacles due to severe side effects and the development of chemoresistance [4]. Research has shown that plant-derived compounds have significant anticancer activities with minimal side effects. Therefore, the discovery of plant-based compounds that are effective against gastric cancer is a promising alternative for cancer prevention and treatment, offering the potential for safer therapeutic options.

Ginseng has been used in traditional medicine for centuries and is recognized for its diverse therapeutic properties, including anticancer, anti-inflammatory, antioxidant, anti-diabetic, cardioprotective, and neuroprotective effects, as well as its role in preventing various diseases in adults [5-9]. These pharmacological effects are attributed to its various bioactive compounds, such as ginsenosides, phenolics, polyacetylenes, peptides, alkaloids, and polysaccharides [10]. Of these, recent studies have shown that ginsenoside 20(S)-Rg3 induces gastric cancer cell apoptosis via the activation of caspase-3, -8, and -9, as well as by regulating Bcl-2 and Bax expression [11]. Ginsenoside Rg5 also suppresses cancer cell proliferation by inducing G2/M phase arrest and promoting apoptosis and autophagy [12]; ginsenoside Rh4 inhibits the SIX1-TGF-β/Smad2/3 signaling axis, thereby blocking gastric cancer metastasis [13]. Therefore, the ingestion of ginsenoside complexes found in ginseng may help prevent and treat gastric cancer by inhibiting its occurrence, metastasis, and growth.

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The types or levels of ginsenosides in ginseng can differ through variation in processing methods, such as steaming and drying [14]. White ginseng, dried without heating, is rich in ginsenosides like Rg1, Rb1, and Rb2 [15]. Red ginseng, steamed at standard boiling temperatures of 100°C and then dried, contains higher concentrations of ginsenosides Rg3, Rg5, and Rk1 [16]. Black ginseng, subjected to the Maillard reaction after nine cycles of steaming and drying, has a remarkably diverse and abundant ginsenoside profile [17]. Accordingly, the pharmacological compounds produced during the transformation of ginseng into black ginseng may exhibit enhanced anticancer efficacy compared to those derived by other methods.

To date, pharmacological evaluations of individual active constituents of black ginseng-derived compounds for gastric cancer have been conducted in both *in vitro* [11-13] and *in vivo* studies [18]. In contrast, the combined pharmacological effects of active components in black ginseng extract (BGE) have been investigated exclusively *in vitro* using the NCI-N87 cell line [16], with no reports from *in vivo* studies. Therefore, this study investigated whether BGE contributes to the prevention and treatment of gastric cancer by examining its effects on inhibiting the proliferation of human gastric cancer cells and growth of solid human gastric tumors *in vivo*.

Materials and methods

Animals

Five-week-old male Athymic NCr-nu/nu mice were obtained from Koatech (Pyeongtaek, Korea). All procedures related to animal housing, handling, and experimentation were conducted in accordance with the Animal Care and Use Guidelines of Kangwon National University and were approved by the Institutional Animal Care and Use Committee (IACUC) (IACUC approval no. KW-240116-1).

Cell culture

Immortalized human AGS cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Ham's F-12K (Kaighn's) medium (Welgene, Gyeongsan, Korea) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Welgene) and 1% (v/v) penicillin-streptomycin solution (Welgene) (herein as referred to AGS cell culture medium). Immortalized human KATO III cells (ATCC) were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Welgene) with the same supplements (herein as referred to KATO III cell culture medium). All cells were maintained in a humidified incubator at 37°C with 5% CO_2, and the culture medium was refreshed at 2-day intervals. On reaching approximately 80% confluence, cells were detached using 0.25% trypsin-EDTA (Welgene) and subsequently reseeded onto 100-mm culture dishes (SPL, Pocheon, Korea). Cells were subcultured at 3-day intervals under the same conditions.

Experimental design

Red ginseng extract (RGE) and BGE (Heaven 351) were donated by Truth & Ginseng Biotechnology Research Cooperation (Jinan, Korea). Stock solutions were prepared by dissolving the extracts in warm distilled water to a concentration of 100 mg/mL. Experiment 1 assessed the cytotoxic or apoptotic effects of BGE on AGS and KATO III cells by measuring cell viability or Annexin V/propidium iodide double positivity following BGE treatment. AGS and KATO III cells were cultured for 48 hours in their respective culture media supplemented with 0, 0.5, 1, 2, 5, or 10 mg/mL BGE, and cell viability was evaluated using a colorimetric alamarBlue® assay. In addition, AGS and KATO III cells were cultured for 48 hours in their respective culture media supplemented with the BGE concentration corresponding to the half maximal inhibitory concentration (IC_{50}) or that resulting in the lowest cell viability, and apoptosis was evaluated by flow cytometry based on Annexin V/propidium iodide double staining. Experiment 2 investigated the antitumor effects of BGE in a gastric tumor-bearing mouse model created by injecting AGS cells into nude mice. The mice were then administered 100 µL of distilled water orally (control), 100 µL of distilled water containing 50 mg/kg RGE, or 100 µL of distilled water containing 50 mg/kg BGE at 1-day intervals for 21 days. During the *in vivo* administration period, tumor volume and body weight were measured every 7 days, and tumor weight was assessed at the end of the 22-day period. Experiment 3 evaluated whether BGE was toxic to the organs of treated mice and the absolute weights and histopathology of the livers and

spleens from AGS cell xenograft nude mice that were treated or not treated with BGE.

Colorimetric analysis

AGS or KATO III cells were seeded in 24-well culture plates (SPL) at a density of 2×10^4 cells per well and incubated for 48 hours at 37°C in their respective culture media containing various concentrations of BGE. Next, cells were rinsed once with Dulbecco's phosphate-buffered saline (DPBS; Welgene) and then treated with 10% (v/v) alamarBlue® (Thermo Fisher Scientific, Waltham, MA, USA) for 4 hours at 37°C. Absorbance was measured at 570 and 600 nm using a microplate reader (Epoch Microplate Spectrophotometer; BioTek Instruments, Winooski, VT, USA).

Flow cytometric analysis

AGS or KATO III cells were plated in 60-mm dishes (SPL) at a density of 1×10^5 cells per dish and incubated for 48 hours at 37°C in their respective culture media supplemented with the BGE concentration corresponding to the half maximal inhibitory concentration (IC_{50}) or that resulting in the lowest cell viability. Following cell scraping, the cells were collected and washed twice with DPBS containing Ca^{2+} and Mg^{2+} (herein as referred to Ca^{2+}/Mg^{2+}-DPBS). The cells were then stained for 30 minutes at room temperature with FITC-conjugated Annexin V (Cat. no. A13199; Thermo Fisher Scientific) diluted 1:50 in Ca^{2+}/Mg^{2+}-DPBS, followed by incubation for 15 minutes at room temperature in Ca^{2+}/Mg^{2+}-DPBS containing 20 μg/ml propidium iodide (Thermo Fisher Scientific). The double-stained cells were sorted using a FACSymphony A3 flow cytometer (BD Biosciences, San Jose, CA, USA) and data were processed using the BD CellQuest Pro software (BD Biosciences).

Preparation of a gastric cancer xenograft mouse model

Fifty microliters of an AGS cell culture medium containing 2×10^6 AGS cells were mixed with 50 μL of Matrigel (Corning, NY, USA). This mixture was then injected subcutaneously into the right and left dorsal flanks of anesthetized mice, with 100 μL delivered at each injection site. When the average tumor volume reached 100 mm^3, 12 mice were randomly assigned to experimental groups, with 4 mice in each group.

Assessment of tumor volume and mouse body, organ, and tumor weights

Tumor volume and mouse body weight were measured prior to euthanasia by cervical dislocation, whereas the weights of tumors and organs were recorded post-euthanasia. The long and short axes of tumors were measured using digital calipers (Traceable Carbon Fiber Calipers; Control Company, Webster, TX, USA), and their volume was calculated using the formula: volume (mm^3) = (long axis × short $axis^2$)/2. The body weights of mice, and organs and tumors collected from mice, were recorded using a scale (Mittler Toledo, Columbus, OH, USA).

Histological analysis

Liver and spleen samples were fixed for 48 hours in 4% (v/v) formaldehyde (Sigma-Aldrich, Burlington, MA, USA) diluted in phosphate-buffered saline (PBS; Thermo Fisher Scientific) at 4°C. After rinsing with PBS, the fixed organs were embedded in paraffin (Leica, Wetzlar, Germany), sectioned into 4-μm-thick slices using a microtome (Leica), and mounted on microscope slides (Matsunami, Kishiwada, Japan). The 4-μm-thick sections were stained with H&E automatically using a TP1020 (Leica) according to the manufacturer's guidelines. The stained tissues were then covered with a coverslip using Permount (Fisher Scientific, Pittsburgh, PA, USA) and examined under an optical microscope (Axio Imager A2; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

All statistical comparisons of the experimental groups were performed using the Statistical Analysis System ver. 9.4 (SAS Institute, Cary, NC, USA). Treatment groups were compared using the least-squares or Duncan method, while the significance of the main effects was determined by analysis of variance (ANOVA) within SAS. A p-value <0.05 was considered to indicate statistical significance.

Results

Experiment 1: In vitro antitumor activity of BGE in human gastric cancer cells

To validate the potential antitumor activity of BGE in human gastric cancer cells, its cytotoxic

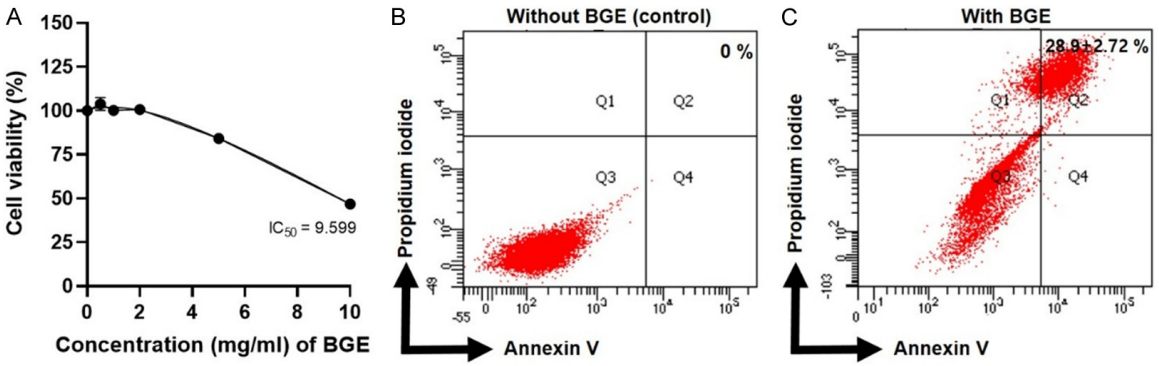

Figure 1. Cytotoxicity and apoptosis induced by BGE in an AGS human gastric cancer cell line. AGS cells were cultured for 48 hours in AGS cell culture medium supplemented with 0, 0.5, 1, 2, 5, or 10 mg/mL BGE. Cell viability was then evaluated using the alamarBlue® assay. To evaluate apoptosis, AGS cells were cultured for 48 hours in AGS cell culture medium either without BGE or with BGE at the concentration corresponding to the half maximal inhibitory concentration (IC_{50}), and Annexin V/propidium iodide double positivity was analyzed by flow cytometry. The IC_{50} was determined to be 9.599 mg/mL BGE (A). Among AGS cells treated with 9.599 mg/mL BGE, 28.9±2.72% of the cells showed Annexin V/propidium iodide double positivity (C), whereas no double-positive cells were detected in AGS cells treated without BGE (B). Data are presented as means ± standard deviation (SD) from three independent experiments. BGE: Black ginseng extract.

and apoptotic effects were evaluated in AGS and KATO III cells, which are widely used models of human gastric cancer cells. In AGS cells, the half maximal inhibitory concentration (IC_{50}) of BGE, representing cytotoxic potency, was determined at 9.599 mg/mL (**Figure 1A**). Cells treated with 0 or 9.599 mg/mL BGE showed 0% (**Figure 1B**) or 28.9±2.72% (means ± SD) Annexin V/propidium iodide double positivity (**Figure 1C**), indicating apoptotic induction. In KATO III cells, cell viability significantly decreased at a BGE concentration ≥2 mg/mL, with higher concentrations leading to an even greater reduction (Supplementary Figure 1A). Correspondingly, 42.8±1.67% (means ± SD) of KATO III cells treated with 5 mg/mL BGE showed Annexin V/propidium iodide double positivity (Supplementary Figure 1C), whereas no double positivity was detected in the 0 mg/mL BGE group (Supplementary Figure 1B). These results demonstrate that BGE induces cytotoxicity and apoptosis in AGS and KATO III cells, indicating its potential as an antitumor agent against human gastric cancer at the cellular level.

Experiment 2: In vivo antitumor activity of BGE in human gastric cancer cells

To evaluate the antitumor effects of BGE on human gastric cancer *in vivo*, BGE was administered orally to AGS cell xenograft nude mice, which are used as a model of human gastric cancer. Tumor volume, tumor weight, and body weight were then measured and compared to those of untreated AGS cell xenograft nude mice. RGE, a known antitumor agent against human gastric cancer, was also administered orally to evaluate its relative efficacy. As shown in **Figure 2A**, from day 7 onward, tumor volume began to increase in the AGS cell xenograft mice. However, treatment with oral RGE or BGE effectively suppressed tumor growth in gastric tumor-bearing mice, leading to significantly smaller tumor volumes than in untreated mice. Furthermore, from day 14 onward, the tumor volume in mice treated with BGE was significantly smaller than in those treated with RGE. Throughout the experiment, no significant changes were observed in body weight among AGS cell xenograft mice treated with oral RGE or BGE (**Figure 2B**). At 22 days post-oral administration, treatment with RGE or BGE caused a significant reduction in tumor weight (**Figure 2C**) and visibly smaller tumors (**Figure 2D**) in the AGS cell xenograft mice compared with the untreated group. These effects were more pronounced with oral BGE than RGE, indicating that oral BGE effectively suppressed the growth of gastric tumors *in vivo* and had significantly stronger antitumor efficacy than RGE.

Experiment 3: Toxicological evaluation of BGE on mouse organs

To evaluate the toxicity of BGE on non-cancerous cells, we analyzed the weights and histopathological features of the liver and spleen

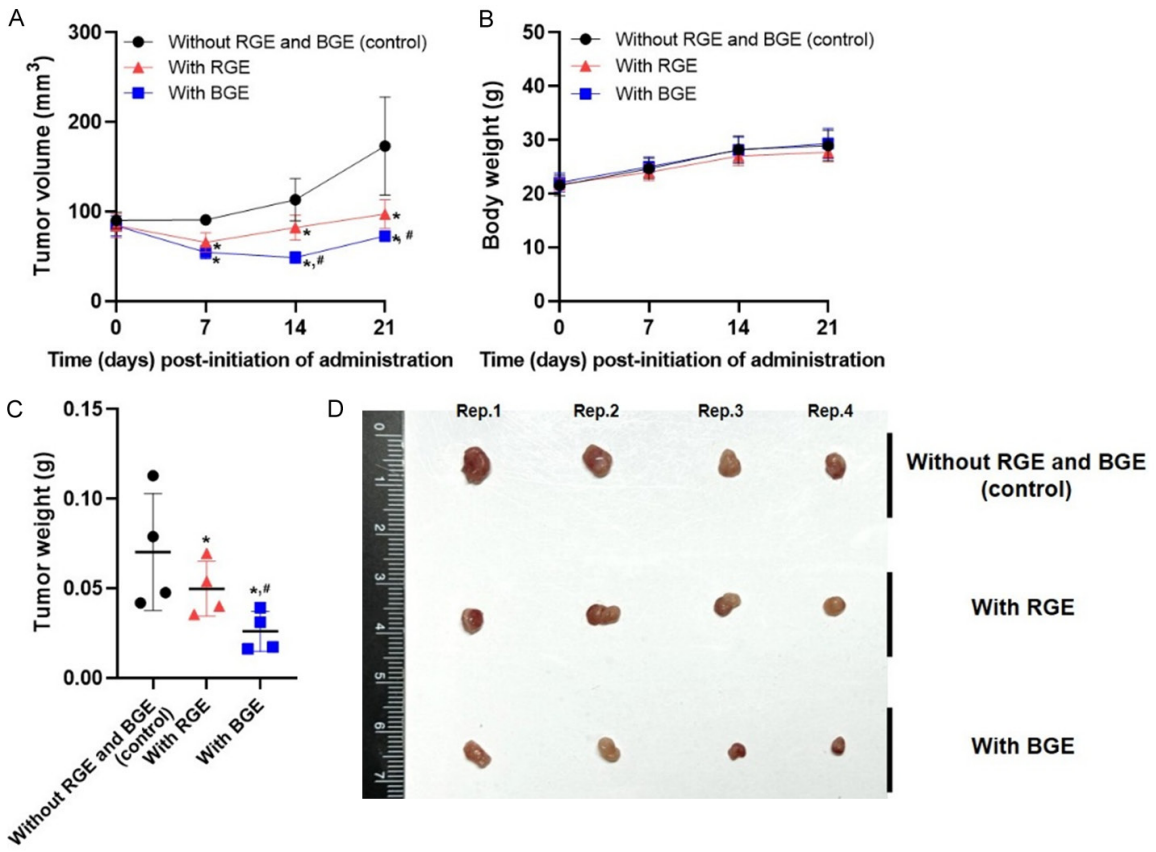

Figure 2. Antitumor effects of BGE in an AGS cell xenograft nude mouse model. AGS cells (2×10^6) were injected into nude mice. When tumor volume reached 100 mm^3, treatment began with oral distilled water (DW; Without RGE and BGE; control), RGE dissolved in DW (With RGE), or BGE dissolved in DW (With BGE) (day 0), and continued daily for 21 days. Tumor volume (A) and body weight (B) were measured at 7-day intervals. On day 22, tumors were harvested, weighed (C), and photographed (D). From day 7 onward, tumor volume increased in AGS cell xenograft nude mice (A). Mice receiving oral RGE or BGE had significantly smaller tumors than the control group (A). From day 14, mice receiving oral BGE had significantly smaller tumors than those treated with RGE (A). Throughout the experiment, no significant differences were observed in body weight (B) between mice receiving RGE versus BGE or the control group. Measurements of tumor weights (C) and sizes (D) at 22 days confirmed that tumors in mice treated with oral RGE or BGE were significantly smaller than those in the control group. Furthermore, tumors in mice treated with oral BGE were significantly smaller than those in mice treated with oral RGE. (D) presents tumors harvested from AGS cell xenograft nude mice treated with BGE (bottom), RGE (middle), or DW (top). (A) and (B) are the mean ± standard deviation (SD) of four independent experiments. Data in (C) are the mean (solid line) of four independent experiments. Rep, replication. $^*P<0.05$ compared with the control group. $^\#P<0.05$ compared with RGE. DW: Distilled water; RGE: Red ginseng extract; BGE: Black ginseng extract.

[19] in gastric tumor-bearing mice after 21 days of oral BGE. No significant differences were observed in liver and spleen weights between the BGE-treated and untreated groups (**Figure 3A**). Histopathologically, liver and spleen tissues from BGE-treated mice exhibited no abnormalities compared with the untreated group (**Figure 3B**). Therefore, BGE does not induce toxicity in non-cancerous cells.

Discussion

Research on anti-gastric cancer compounds derived from medicinal plants, which can minimize side effects, is an important approach to alleviate the non-specific toxicity of and resistance to cancer chemotherapy. Black ginseng is regarded as a superior medicinal plant in terms of its anticancer compound composition and content. Therefore, we assessed the antitumor efficacy of BGE on gastric cancer tumors. *In vitro* analysis revealed that BGE was cytotoxic and apoptotic in both AGS and KATO III gastric cancer cells. Furthermore, *in vivo*, the injection of AGS cells into mice subcutaneously significantly delayed tumor growth without causing systemic toxicity. Notably, the group treated with oral BGE had significantly smaller

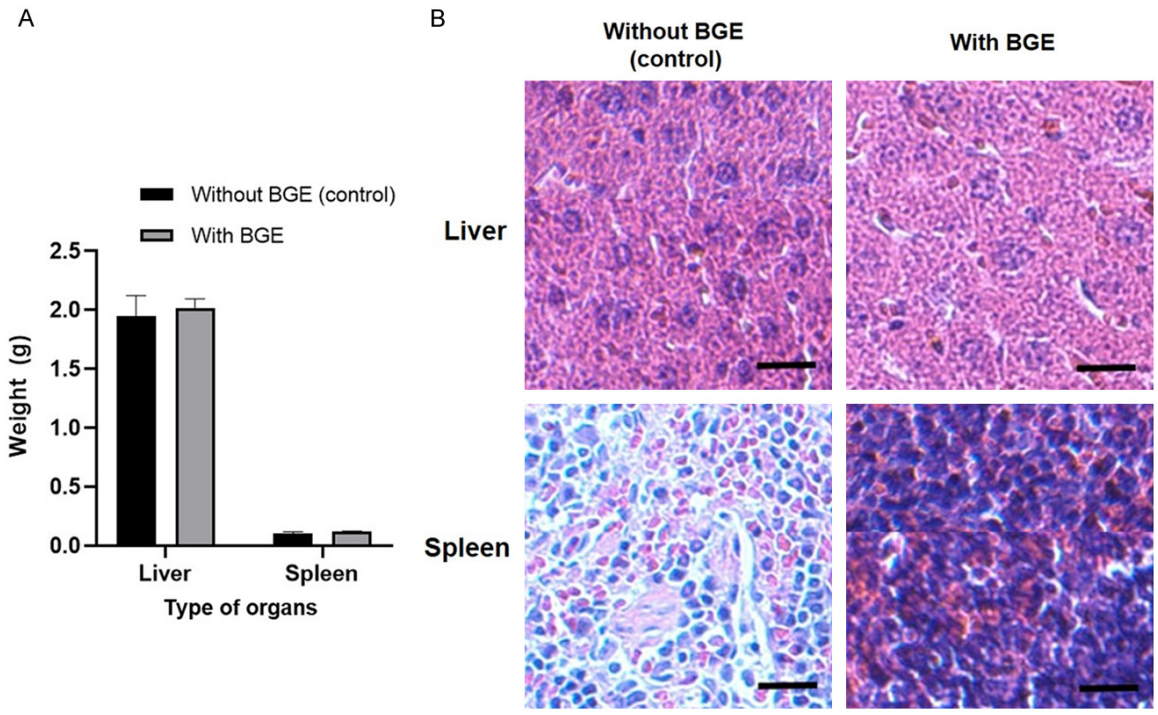

Figure 3. Absolute weight and histopathological analysis of liver or spleen from AGS cell xenograft nude mice treated with oral BGE. AGS cells (2×10^6) were inoculated into nude mice. When tumor volume reached 100 mm^3, treatment with oral DW (control) or BGE dissolved in DW (With BGE) was initiated and continued daily for 21 days. At necropsy, livers and spleens were excised from mice and weighed. Organs were then sectioned into tissue slices, which were fixed, processed, paraffinized, and stained with hematoxylin and eosin. No significant differences were observed in liver or spleen weights between mice treated with BGE and the control group (A). Representative micrographs did not reveal any histopathological BGE-related toxic effects in the livers or spleens of mice treated with BGE compared with the control group (B). Data in (A) are the means ± standard deviation (SD) of three independent experiments. Scale bar in (B) = 50 μm. DW: Distilled water; BGE: Black ginseng extract.

tumors than the group treated with oral RGE, suggesting that BGE has superior anticancer activity. BGE appears to be a promising therapeutic alternative for gastric cancer.

Ginsenoside 20(S)-Rg3 [11] and Rg5 [12] induces apoptosis in gastric cancer cells via the co-promotion of PARP cleavage, activation of caspase-3, -8, and -9, down-regulation of Bcl-2, and up-regulation of Bax. Ginsenoside Rg5 induces autophagy by arresting the G2/M phase by regulating cell cycle regulatory proteins, up-regulating LC3B-II, Beclin-1, Atg-5, and Atg-12, and down-regulating p62 [12]. Moreover, ginsenoside Rh4 significantly inhibits the growth, proliferation, epithelial-mesenchymal transition (EMT), and invasion of gastric cancer cells by blocking the activation of the SIX1-TGF-/Smad2/3 signaling axis both *in vitro* and *in vivo* [13]. In this study, BGE, which contains ginsenosides 20(S)-Rg3, Rg5, and Rh4 [17], exhibited antitumor effects against gastric cancer at both the cellular and histological levels. Accordingly, the antitumor effects of BGE may be due to this combination of ginsenosides, which promote cell apoptosis and autophagy, in addition to inhibiting cell growth, proliferation, EMT, and invasion.

Increasing the number of steaming and drying cycles of ginseng significantly alters the composition of ginsenosides by increasing ginsenosides (Rg3, Rg5, and Rk1) with molecular weights below 800 Da, and decreasing larger ginsenosides (Rb, Rc, and Rd) [20]. Thus, black ginseng, which undergoes more steaming and drying than red ginseng, contains higher concentrations of Rg3, Rg5, and Rk1, but lower levels of Rb, Rc, and Rd than red ginseng [21]. In various cancers, ginsenosides Rb, Rc, and Rd have extremely weak anticancer efficacy, whereas Rg3, Rg5, and Rk1 have significantly stronger effects [22]. Accordingly, the enhanced anticancer effects of BGE against

human gastric cancer, compared with RGE, may be attributed to the higher concentrations of ginsenosides Rg3, Rg5, and Rk1 with stronger anticancer activity.

The primary physiological mechanisms contributing to the onset and progression of gastric cancer are inflammation [23] and oxidative stress [24], with *Helicobacter pylori* infection [25] and smoking [26] identified as major risk factors. Tumor necrosis factor-α (TNF-α)-inducing protein (Tipα) secreted by *H. pylori* interacts with the cell surface receptor nucleolin, triggering the expression of various pro-inflammatory cytokines, including TNF-α, interleukin (IL)-8, IL-1α, IL-1β, and IL-6 in the gastric mucosa [27]. This interaction promotes the EMT, accelerating the development of gastric cancer [27]. Smoking stimulates gastric acid secretion [28], while suppressing the release of protective prostaglandins in the gastric mucosa [29]. As a result, the gastric mucosa becomes susceptible to oxidative stress, leading to cellular damage and inflammation and further increasing vulnerability to *H. pylori* infection, a major risk factor for gastric cancer. Recent studies have confirmed that BGE inhibits the expression of inflammatory mediators, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α [30]. Furthermore, BGE protects cells from oxidative damage by scavenging reactive oxygen species (ROS), maintaining redox balance, and activating the antioxidant defense system [31]. These findings suggest that BGE exerts anti-inflammatory and antioxidant effects by modulating inflammation-related metabolic pathways and reducing oxidative stress. Accordingly, the anticancer effects observed in this study may be attributed to the dual action of BGE reducing inflammation and combating oxidative damage, indicating that regular consumption of BGE might help prevent the development of gastric cancer.

In summary, BGE had anticancer effects against gastric tumors at both the cellular and histopathological levels, with no signs of toxicity in major organs. These findings highlight the potential of BGE as a promising anti-gastric cancer agent, as well as its potential role as adjunctive therapy and preventing gastric cancer.

Disclosure of conflict of interest

None.

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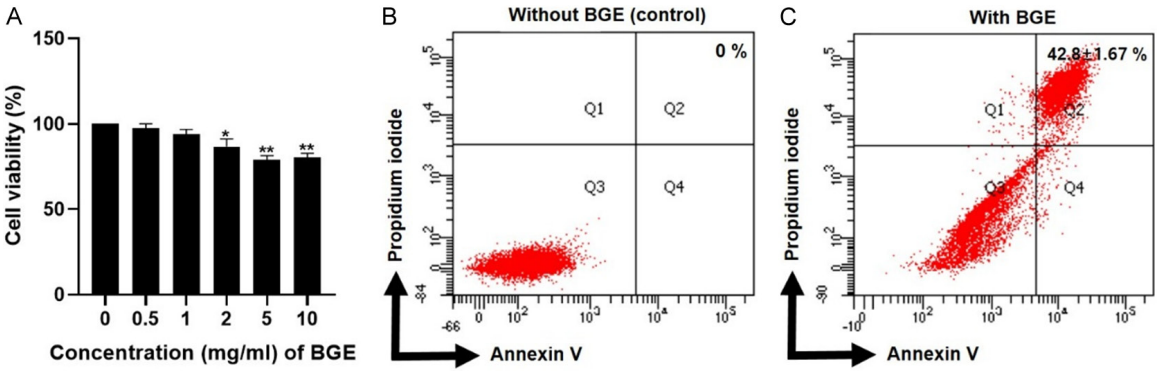

Supplementary Figure 1. Cytotoxicity and apoptosis induced by BGE in KATO III human gastric cancer cell line. KATO III cells were cultured for 48 hours in KATO III cell culture medium supplemented with 0, 0.5, 1, 2, 5, or 10 mg/mL BGE. Cell viability was then evaluated using the alamarBlue® assay. To evaluate apoptosis, KATO III cells were cultured for 48 hours in KATO III cell culture medium either without BGE or with BGE at the concentration resulting in the lowest cell viability, and Annexin V/propidium iodide double positivity was analyzed by flow cytometry. Significantly the lowest cell viability was observed at 5 and 10 mg/mL BGE (A). Among AGS cells treated with 5 mg/mL BGE, 42.8±1.67% of the cells showed Annexin V/propidium iodide double positivity (C), whereas no double-positive cells were detected in KATO III cells cultured without BGE (B). Data are presented as means ± standard deviation (SD) from three independent experiments. $^{*,**}P<0.05$. BGE: Black ginseng extract.