Original Article

Antitumor Activities and Apoptosis-regulated Mechanisms of Fermented Wheat Germ Extract in the Transplantation Tumor Model of Human HT-29 Cells in Nude Mice[*]

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Abstract

Objective   A subcutaneous transplantation tumor model of human HT-29 cells in nude mice was established to evaluate anticarcinogenic activities, and the apoptosis-regulated mechanism effect of aqueous extract of fermented wheat germ with Lactobacillus plantarum dy-1 (LFWGE).

Methods   The HT-29 cells were transplanted via subcutaneous injection of 1×10^7 cells into the right flank of each nude mouse. Then, nude mice were treated for 30 d with LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and for 7 d with 5-fluorouracil (5-FU, 25 mg/kg/d) by gavage and intraperitoneal injection, respectively. An inhibition of tumor growth was observed.

Results   Tumor volume and weights decreased significantly in both groups of nude mice treated with LFWGE. In addition, the cell apoptosis rate of the LFWGE group (2 g/kg/d, 60.1%±4.4%; 1 g/kg/d, 58.6%±6.9%) was significantly higher than that of the control group (11.5%±1.6%) and 5-FU group (32.1%±3.5%) as measured by the TUNEL assay. Moreover, the real-time fluorescent quantitative PCR and Western blot method further confirmed these enhancing apoptosis and growth inhibition effects. The involvement of LFWGE in inducing apoptosis was confirmed by the expression of Bax, Bcl-2, Caspase-3, and CyclinD1.

Conclusion   The results showed that LFWGE could induce subcutaneous transplantation tumor apoptosis in nude mice and could be as a natural nutrient supplements or chemopreventive agent in the treatment of human colon cancer.

Key words: Fermented wheat germ extract; Nude mice; Antitumor; Apoptosis; Western blot; Human HT-29 cells

INTRODUCTION

In its 'World Cancer Report', the World Health Organization (WHO) revealed that China accounted for almost half of the world's cancer incidences in 2012. Morbidity and mortality continue to show an upward tendency. Colon cancer is currently one of the most common malignancies and leading causes of cancer-related deaths. Surgery, chemotherapy, radiotherapy and targeted therapy (immunotherapy, gene therapy, angiogenesis inhibitors, and others) are approaches to treating cancer. Although it is the most common daily treatment, drug treatment is problematic due to its side effects. Accumulating evidence from the epidemiology, human medicine and nutrition fields

has indicated that dietary agents can safely regulate physiological function and enhance anticarcinogenic activity[1-2]. In addition, natural products have become more popular for the prevention and treatment of cancer[3-5].

Wheat germ (embryonic axis and scutellum) has a total seed weight of about 2.5%-3.8% and is an important byproduct of the flour milling industry[6]. It is systematically removed during milling because it adversely affects the shelf life and quality of flour. However, its various physiological active ingredients lend it broad application prospects and developmental value. Several studies have recently shown that *Saccharomyces cerevisiae* fermented wheat germ extract (MSC; trade name: Avemar) possesses antioxidant, anti-inflammatory[7] and anti-carcinogenic activities in various cancer models, such as testicular, colon, non small cell lung, melanoma, leukemia and gastric cancers[8-9].

Studies involving *Lactobacillus plantarum* fermented wheat germ have raised concerns in recent years[10-12]. In 2013, lactic acid bacteria isolated from wheat germ were characterized and selected based on technological performance to be used as starters for fermenting wheat germ, and an Italian researcher found that the fermented wheat germ extract could inhibit the growth of the colorectal cancer cell lines HT-29, HCT-8, and DLD-1[12]. Our previous *in vitro* studies reported that fermented wheat germ extract with *Lactobacillus plantarum* dy-1 (LFWGE) had a strong inhibitory effect on HT-29 cell proliferation and apoptosis related to chemical composition, including some functional proteins and quinines[13]. Despite the potential value of a relevant *in vitro* study for human colon cancer, no animal experiment results have been reported.

Based on our preliminary *in vitro* research, further *in vivo* experiments are required. This study used the transplantation tumor model of human HT-29 cells in nude mice to observe the antitumor activities of LFWGE *in vivo*. It investigated the relationship between this apoptosis and expressions of Bcl-2, Bax, CyclinD1, and Caspase-3 that will be able to provide a theoretical and methodological basis for its future clinical application.

MATERIALS AND METHODS

We obtained 40 female BALB/c nude mice aged 4-6 weeks and weighing 16-18 g from Shanghai Silaike Laboratory Animal Limited Liability Company. The code number of the animals was SCXK (SU) 2012-0002. The nude mice were caged individually under specific-pathogen free (SPF) conditions in the Laboratory Animal Research Center of Jiangsu University (LARC, Zhenjiang, China) at a temperature of (22±2) °C and a relative humidity of 40%-60% and artificially illuminated on an approximate 12-h light/dark cycle. The air exchange rate was about 18 times per hour. All of the nude mice were provided with food and sterile water ad libitum. The Shanghai Medical Experimental Animal Care Commission approved the experimental protocol.

Fresh wheat germ was purchased from Shandong Yongle of China. The lactobacillus strain *Lactobacillus plantarum* dy-1 which we had previously isolated from pickles. Its preservation number was CGMCC NO. 6016 in the Chinese common microbe preservation administration center. HT-29 colon cancer cells were obtained from the Shanghai Institutes for Biological Sciences (SIBS) of the Chinese Academy of Sciences (CAS). Fetal bovine serum and McCoy's 5A medium were purchased from GIBCO. FBS, MTT and DMSO were purchased from Evergreen. 5-fluorouracil (5-FU) was purchased from Jiangsu Deyuan Pharmaceutical Co. Ltd. All of the other biochemical reagents and solvents involved were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) and were analytical or chromatographic grade. A fluorescence quantitative PCR-related kit for treasure biological (Takara) products, a first antibody and secondary antibodies were purchased from Abcam Company.

Extract Preparation

Lactobacillus plantarum dy-1 was used as the starter culture in the direct vat set fermentation. It was cultivated in MRS, treated with a protective agent (20% skim milk, 10% fucose, 14% monosodium glutamate, 6% sorbitol, and 6% Vitamin C) and lyophilized to powder. The wheat germ was crushed and passed through a 100-mesh sieve. Two hundred grams of wheat germ powder, 1.4 L of distilled water and 4 g of freeze-dried fungus powder containing *Lactobacillus plantarum* dy-1 (cell density $4×10^8$ cfu/g) were shaken and incubated at 30 °C for 24 h in a microbiological incubator. After fermentation, the mixture was centrifuged at 12,000 g for 15 min at 4 °C using a refrigerated centrifuge (Jouan, France). The supernatant was then freeze-dried into a powder (LFWGE) with a vacuum freeze dryer (Marin Christ, Germany). These steps were repeated six times. The freeze-dried samples were stored in

sealed containers at -20 °C for further use.

Cell Culture

Human colon cancer cell strain HT-29 was thawed and cultured as monolayers of up to 80% confluence in McCoy's 5A supplemented by 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin. The insemination and embryo cultures were performed in a CO_2 incubator (Forma 310, Thermo Fisher Scientific, MA, USA) under an atmosphere of 5% CO_2 in humidified air at 37 °C. Cell counts were determined using a micro-cell counter CC-108 (Sysmex, Kobe, Japan). Cells in a logarithmic phase of growth were used for the study and are described as follows.

Establishment of the Transplantation Tumor Model and Grouping

The HT-29 cells were transplanted via subcutaneous injection of 1×10^7 cells into the right flank of each nude mouse. One week after transplantation, tumors had grown to a volume of approximately 20 mm^3 with a model success rate of 100%. The 40 nude mice models were equally randomized and divided into four experimental groups, including high- and low-dose LFWGE, 5-FU and control groups.

The high- and low-dose LFWGE groups were treated with 2 g/kg and 1 g/kg LFWGE by gavage once a day for 30 d, respectively. The control group received equivalent amounts of normal saline in the same way. The 5-FU group received intraperitoneal injections of 25 mg/kg/d continued for 7 d at first, and treated with normal saline by gavage once a day for 30 d in order to eliminate the effect of gavage (Table 1).

Table 1. The Different Groups and the Administration Dosages

Group	Administration Dosages	Days
Control	Normal saline, 15 mL/kg /d	30
5-FU	5-FU, 25 mg/kg/d	7[*]
Low-dose LFWGE	LFWGE, 1 g/kg /d	30
High-dose LFWGE	LFWGE, 2 g/kg/d	30

Note. [*]The 5-FU group received intraperitoneal injections of 25 mg/kg/d continued for 7 d at first, and treated with normal saline by gavage once a day for 30 d.

The volume of a tumor was calculated using the following formula: tumor volume (mm^3) V = ($a^2\times b$)/2, where a = the shortest diameter and b = the longest diameter of the tumor (in mm), forming the tumor growth curve. In addition, the tumor inhibitory rate = (1-mean tumor weight of the treatment group/mean tumor weight of the control group) × 100%. At 30 d, 10 animals in each group were sacrificed. Tumor tissue was excised by scalpel for shape observation and gravimetry. In addition, part of the tumor tissue and liver specimens were taken for routine pathology and electron microscopy observation. The rest of the tumor tissue was frozen in liquid nitrogen and kept at -80 °C for further use.

Assessment of Apoptosis by TUNEL

TUNEL staining was performed using the ApopTag kit (Oncor, Purchase, NY, USA). Sections (4 µm) were briefly deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol, boiled in Citra (Biogenex, San Raman, CA, USA) for 10 min and digested in 0.5% pepsin for 60 min at 37 °C. Endogenous peroxidase was then blocked in 3% hydrogen peroxide. Three different dilutions (1:7, 1:11, and 1:16) of terminal deoxynucleotidyl transferase (TdT) in reaction buffer (containing a fixed concentration of digoxigenin-labeled nucleotides) were applied to serial sections for 1 h at 37 °C. The slides were then placed in a stop/wash buffer for 10 min. Following the washes, a prediluted anti-digoxigenin peroxidase-conjugated antibody was applied for 30 min. Apoptotic cells were detected after incubation in the 3,3-diaminoben-zidine (DAB) chromogen (DAKO, Carpinteria, CA, USA) for approximately 6 min, and the slides were counterstained with methyl green (Sigma, St. Louis, MO, USA). Ten high power fields were selected for each slice, and the number of positive cells was counted for every 1,000 cells. The apoptosis rate (%) =number of positive apoptosis cell/1000×100%.

Real-time Fluorescence Quantitative PCR Analysis

Tumor tissue was pestled in liquid nitrogen and lysed in Trizol reagent. Total RNA was prepared according to the manufacturer's instructions (Takara, Japan). To remove the genomic DNA, 5 µg of total RNA was treated with 5U RNAse-free DNAseI for 30 min at 37 °C. After the DNAse treatment, the RNA was incubated at 65 °C for 10 min. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Quantitative PCR was used to generate RNA with two sharp ribosomal 18S

and 28S bands. First-strand cDNA synthesis was carried out on 2 μg of the total RNA from each sample using the PrimeScript® RT Master Mix (Takara, Japan) first-strand synthesis kit for RT-PCR according to the manufacturer's instructions. Experimental wells containing 25 μL of SYBR Green PCR Master Mix (Takara, Japan) were run using north tube plates. Quantitative PCR was conducted on the iCycler according to the SYBR Green method. Forward and reverse primers were designed using the tools available on the MIT Whitehead Institute website. The following primer sequences were used: Caspase-3(F)AACCTCAGGGAAACATTCAG, Caspase-3 (R)GGCTCAGAAGCACACAAAC, Bax(F)AGGATCGAGCA GGGCGGCGAATG, Bax(R)GACACTCGCTCAGCTTCTTG G, Bcl-2(F)ATTTCCTGCATCTCATGCCAAGGG, Bcl-2(R) TGTGCTTTGCATTCTTGGACGAGG, CyclinD1(F)CATGG AACACCAGCTCCTGTG, CyclinD1(R)GTTCATGGCCAGC GGGAAGAC, β-Actin(F)AGCGAGCATCCCCCAAAGTT, and β-Actin(R)GGGCACGAAGGCTCATCATT. Quantitative PCR amplification for all of the Caspases included pre-incubation at 94 °C for 6 min, followed by 38 cycles at 95 °C for 5 s, 57 °C for 45 s, and 72 °C for 30 s. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method[14].

Western Blotting

The protein concentration of the tissue extracts was determined by BCA protein assay. An aliquot of the tumor tissue extract lysed using cell lysis buffer [0.5 mol/L Tris-Hcl pH-7.5, 0.15 mol/L of NaCl, 0.001 mol/L of EDTA, 2 μg/mL of Aprotinin, 0.001 mol/L of phenylmethanesulfonyl fluoride (PMSF)] and protease inhibitor cocktail (purchased from Bi Yuntian) was subjected to SDS-PAGE on 10%-12% Tris-glycine gel. The separated proteins were transferred onto PVDF membrane (Millipore) and were probed with Bcl-2, Bax, Cyclin D1, Caspase-3 antibodies (Abcam Inc., Cambridge, MA, USA) using peroxidase-conjugated appropriate secondary antibody (Abcam Inc., Cambridge, MA, USA). Signals were visualized using the Chemilumniscence HRP detection system (Millipore) on Versa Doc (Bio-rad). Membranes were stripped and re-probed with a β-actin antibody (Abcam Inc., Cambridge, MA, USA).

Statistical Analysis

The statistical analysis was conducted using SPSS software version 17.0 (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was conducted to test for differences between the means.

RESULTS

Tumor Cell Proliferation in Nude Mice

The main composition (% Dry-Weight) of fermented wheat germ extract with *Lactobacillus plantarum* dy-1 (LFWGE) and wheat germ extract (WGE) The LFWGE are shown in Table 2[13]. The inhibitory effects of LFWGE and 5-FU on the growth of tumors in nude mice were observed and the inhibitory rates were calculated. Figure 1 shows the tumor volume measured at intervals of 3-4 d. After 30 d, the tumor volumes of the control, 5-FU, high-dose LFWGE and low-dose LFWGE groups were (1,071.03±159.05), (763.86±108.63), (659.75±204.17), and (688.37±198.37) mm^3, respectively. Compared with the control group, the tumors of the LFWGE and 5-FU groups shrank significantly ($P<0.05$), especially in the high-dose LFWGE group. The results showed that LFWGE had inhibitory effects on the transplantation tumor of human HT-29 cells in BALB/c nude mice. At the end of the experiment, all of the tumors were isolated from the nude mice and weighed. The tumor weights in the nude mice from the control, 5-FU, high-dose LFWGE and low-dose LFWGE groups were (0.916±0.018), (0.640±0.023), (0.534±0.015), and (0.543±0.015) g, respectively, as shown in Figure 2. The final tumor weights of the LFWGE and 5-FU groups and the final tumor volume decreased compared with those of the control group. Treatment with LFWGE and 5-FU significantly inhibited tumor growth. The inhibitory effects of the 5-FU, high-dose LFWGE and low-dose LFWGE groups were 30.08%, 41.72%, and 40.70%, respectively. LFWGE exhibited a stronger ability to inhibit tumor growth than 5-FU. However, LFWGE inhibited HT-29 cell growth was no dose-dependent manner.

Table 2. Main Composition (% Dry-Weight) of LFWGE and WGE

Composition	WGE	LFWGE
2, 6-dimethoxybenzoquinone (μg/g)	33.85±2.85	181.10±4.56
Protein (%, w/w)	32.90±0.57	34.36±0.23
Total phenols (mg gallic acid /g)	20.81±0.11	28.48±0.16
Total sugar (%, w/w)	43.05±1.23	33.04±1.03
Lactic acid (mg/g)	0	27.7±1.2

Note. The results are expressed as mean±SD, *n*=3.

Figure 3 shows the tumors, which were isolated from the nude mice on day 30. All of the tumors had spherical or ellipsoid shapes and smooth surfaces with dendritic protrusions. The surrounding tissues had very clear boundaries and easily or mildly adhered to the skin and subcutaneous tissue. They exhibited a complete coating, a fleshy red surface, a flexible fish-shaped profile texture and occasional white bean dregs. The figure also reveals that LFWGE significantly inhibited tumor growth and proliferation effects.

Moreover, Figure 4 presents the morphology of tumor tissues in different treatment groups' nude mice. From Figure 4, it is clearly noted that significant changes in tumor tissues morphological could be observed when comparing control with 5-FU,
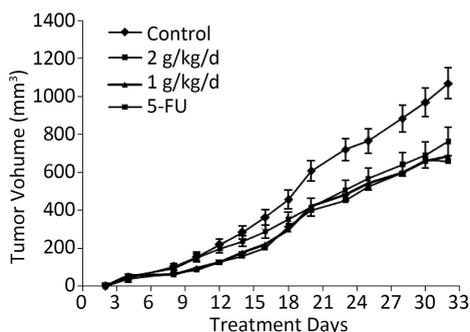
LFWGE (1 g/kg/d) and LFWGE (2 g/kg/d). The results indicated HT-29 cells distributed dense and nuclear atypia is big in tumor tissues of control. However, HT-29 cells were broken, loosed and apoptosis in tumor tissues treatment with LFWGE and 5-FU.

Histological Examination

To examine the effects of LFWGE on liver, the morphology of liver tissues was assessed using electron microscopy. Figure 5 presents the morphology of liver tissues in different treatment groups' nude mice. From Figure 5, it is clearly noted that there were no significant difference in the morphology of liver tissues of all groups. Besides, no animals showed signs of liver metastasis, hyperemia and edema. Moreover, organ index was determined

Figure 1. Growth curve of tumor volume in nude mice transplantation tumors during treatment with LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d). n=10. Values represent the mean±SD.
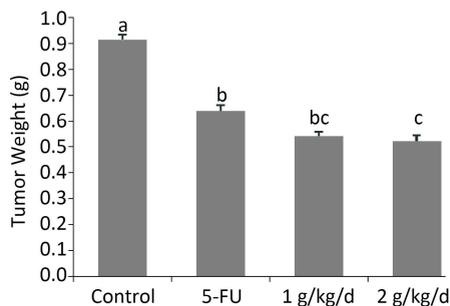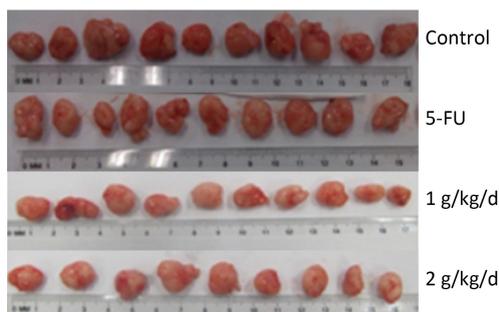

Figure 2. Tumor weights in nude mice transplantation tumors during treatment with LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d). On day 30, the mice were sacrificed and the tumor weights were measured. n=10. Values represent mean±SD. Different letters indicate a significant difference ($P<0.05$).

Figure 3. Picture of tumors in nude mice transplantation tumors during treatment with LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d). n=10.
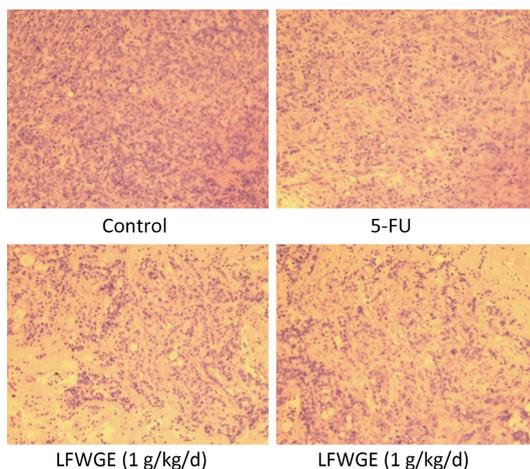

Control   5-FU

LFWGE (1 g/kg/d)   LFWGE (1 g/kg/d)

Figure 4. Effects of LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d) on the morphology of tumor tissues in HT-29 xenograft (×200).

(organ weight/nude mice weight), no significant changes in heart, lung and kidney could be observed when comparing control with 5-FU, LFWGE (1 g/kg/d) and LFWGE (2 g/kg/d). These results indicated that LFWGE was no toxicity to liver, heart, lung, and kidney.

Tumor Apoptosis in Nude Mice

The cell apoptosis rate was determined via TUNEL staining to reveal how LFWGE and 5-FU inhibited tumor growth. The nuclei of normal tumor cells were colored dark blue by hematoxylin re-dyeing. According to Figure 6, the following apoptotic features of apoptotic positive cells were found in the TUNEL staining: a shrinking cell body, nuclear pyrosis, chromatin condensation and tan or brown granules. The cell apoptosis rates of the control, 5-FU, high-dose LFWGE and low-dose LFWGE groups were (11.5±1.6)%, (32.1±3.5)%, (60.1±4.4)%, and (58.6±6.9)%, respectively (Figure 6). The cell apoptosis rate of the LFWGE group was obviously higher than those of the control and 5-FU groups ($P<0.05$). LFWGE induced subcutaneous transplantation tumor apoptosis. These results are consistent with those observed in previous *in vitro* experiments[13].

Apoptotic Effect of LFWGE via Caspase-3 and Bax Activation and Down-regulating Bcl-2, Cyclin D1 Expression in HT-29 Colon Cancer Cells

To explore the mechanism of LFWGE pro-apoptotic and anti-proliferative activities, fluorescen-

nce quantitative PCR was performed to examine the mRNA expression of Bax, Bcl-2, Cyclin D1 and Caspase-3 in tumor tissue of nude mice in all of the groups. The RNA purity was quantified by spectrophotometer with an OD260/280 ratio of 1.8 to 2.0 for all specimens. The RNA integrity was determined by 1% agarose gel electrophoresis, yielding two sharp ribosomal 18S and 28S bands. Figure 5 shows the results.

Compared with the control group, the mRNA-relative contents of CyclinD1 were 0.907, 0.431 and 0.422 in the tumor tissue of the 5-FU, high-dose LFWGE and low-dose LFWGE groups, respectively. Furthermore, the mRNA relative contents of Bax were 1.116, 1.532, and 1.597, respectively; the mRNA relative contents of Bcl-2 were 0.997, 0.435, and 0.660, respectively; and the mRNA relative contents of Caspase-3 were 1.557, 2.205 and 2.054, respectively (Figure 7). The mRNA expressions of Bax and Caspase-3 in the LFWGE group increased significantly compared with those in the control and 5-FU groups after 30 days ($P<0.05$). Moreover, the mRNA expressions of Bcl-2 and CyclinD1
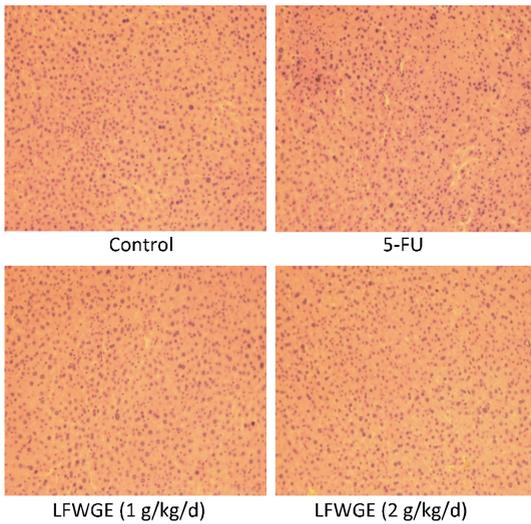

Figure 5. Effects of LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d) on the morphology of liver tissues in HT-29 xenograft (×200).
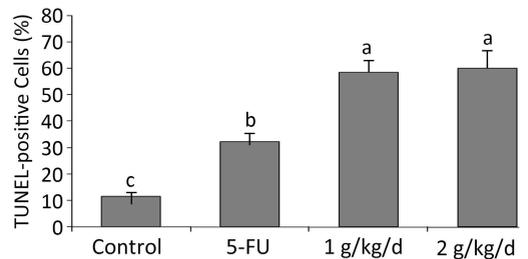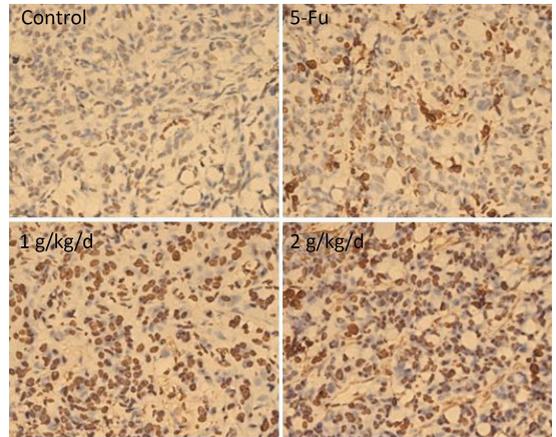

Figure 6. Effects of LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d) on nude mice transplantation tumors apoptosis. *n*=6. Values represent mean±SD. Different letters indicate a significant difference ($P<0.05$).

decreased significantly (P<0.05). LFWGE may have promoted tumor apoptosis by up-regulating the mRNA expressions of Bax and Caspase-3 and down-regulating the mRNA expressions of Bcl-2 and CyclinD1.

To further explore the mechanism of LFWGE pro-apoptotic and anti-proliferative activities, Western blotting was performed to examine the protein expressions of Cyclin D1, Bax, Bcl-2, and Caspase-3 in tumor tissue of nude mice.

As shown in Figure 8, under the LFWGE treatment, the protein expressions of Bcl-2 and CyclinD1 were significantly down-regulated compared with the control group (P<0.05) and the protein expressions of Bax and Caspase-3 were significantly up-regulated (P<0.05). However, under the 5-FU treatment, the protein expressions of Bcl-2, Bax and CyclinD1 showed no significant difference (P>0.05) and that of Caspase-3 was significantly up-regulated (P<0.05). Different functional components of LFWGE may play important roles in promoting apoptosis pathways, indicating the differences between LFWGE and 5-FU. In this study, 5-FU mainly activated the expression of Caspase-3 to achieve apoptosis. Studies have reported that Caspase-3 plays an important role in colorectal cancer. Nevertheless, 5-FU is used as an intermediate to activate Caspase-3 to apoptosis[15]. In renal cell carcinoma, 5-FU can activate Caspase-3, 6, 8, 9 simultaneously[16]. The results of mRNA expressionand protein amount of CyclinD1 and Bax were some differences, maybe different experiments existed differences in operation and mRNA expression had not always been the same as that of protein expression. But the overall trend is the same.

Finally, the Western blotting results were consistent with the quantitative PCR results. LFWGE inhibited proliferation and induced apoptosis mainly by down-regulating the CyclinD1 and Bcl-2 gene expressions and up-regulating the Bax and Caspase-3 gene expressions.
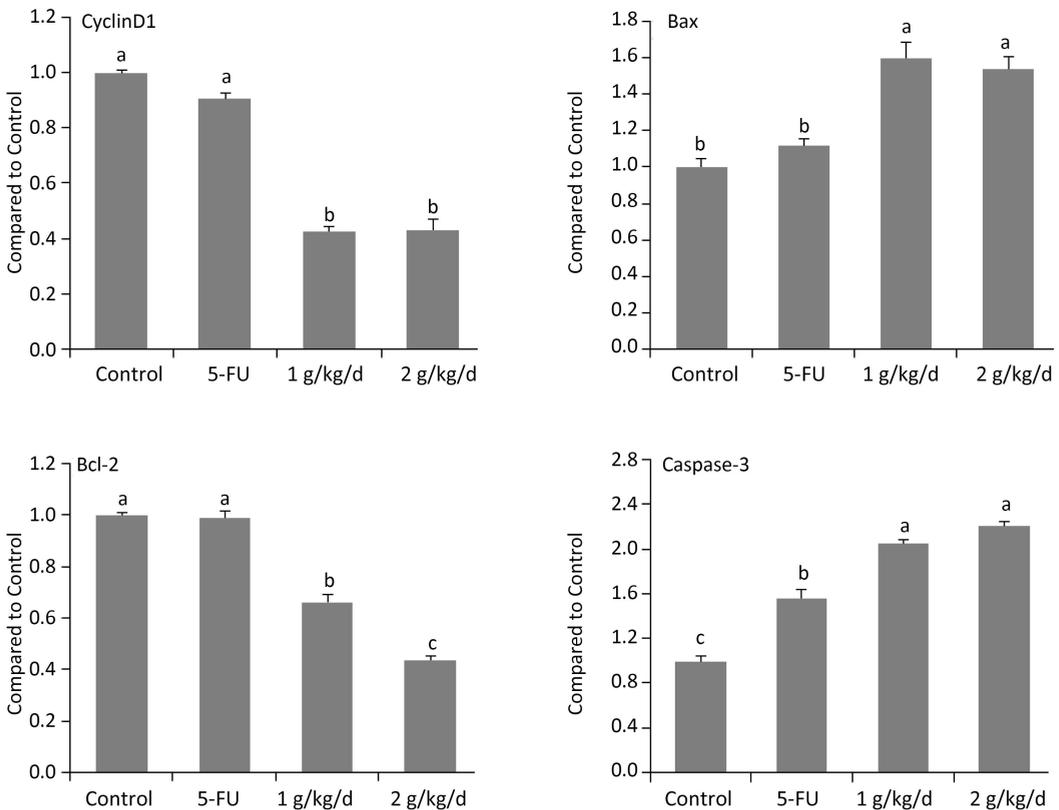

Figure 7. CyclinD1, Bax, Bcl-2 and Caspase-3 mRNA expression analysis in nude mice transplantation tumors during treatment with LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d). n=6. Values represent the mean±SD. Different letters indicate a significant difference (P<0.05).

DISCUSSION

Wheat germ that contains beneficial nutritional values is frequently used in human food supplements, breakfast cereals, nutri-bars, and various fiber drink mixtures. Fermenting wheat germ with baker's yeast has a surprising immunostimulatory and metastasis inhibiting effect as a nutritional supplement for cancer patients.

This study investigates the effect of anticancer and the biochemical mechanism of the action of LFWGE in a subcutaneous transplantation tumor model
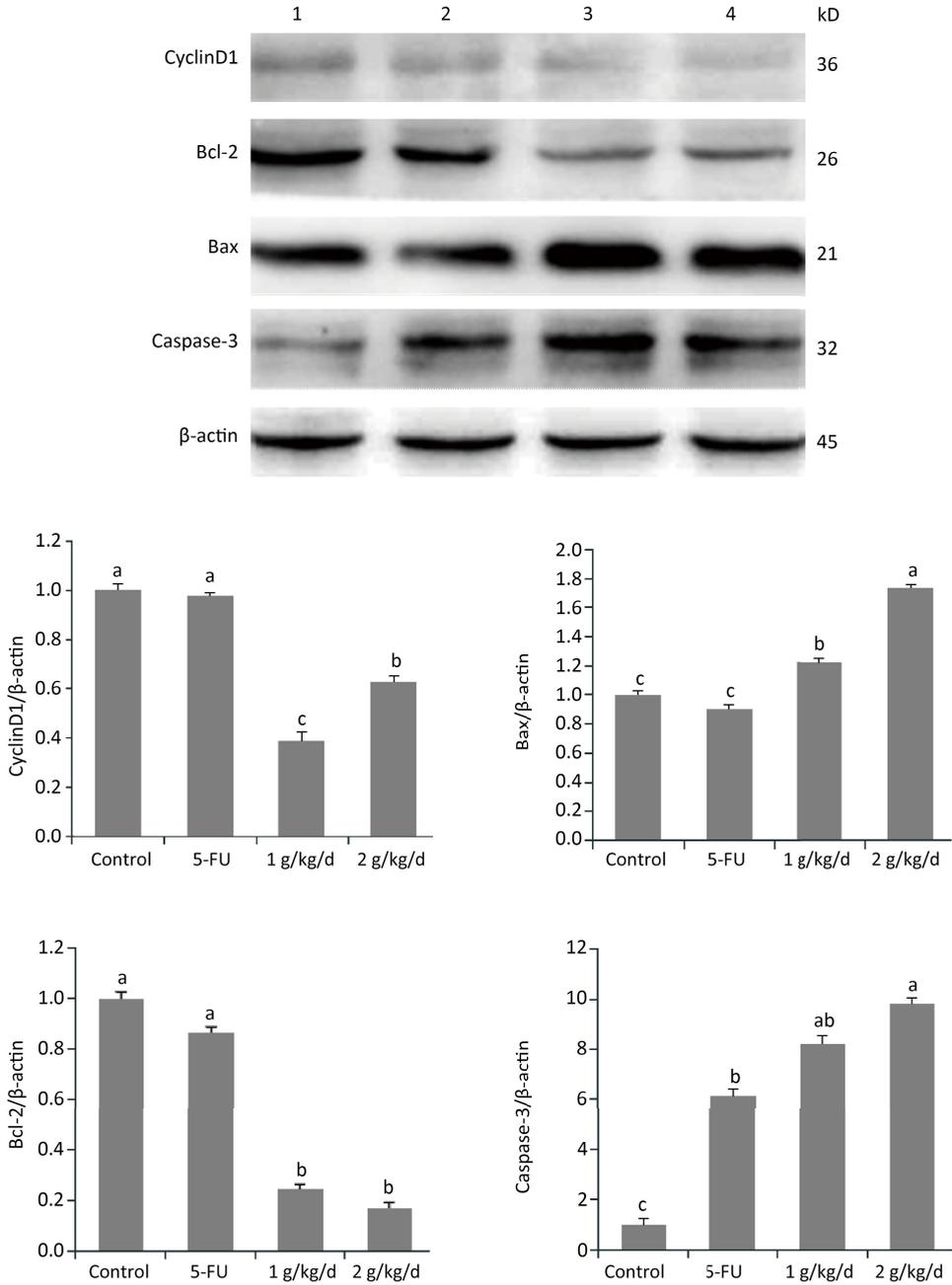

Figure 8. Expressions of CyclinD1, Bax, Bcl-2, and Caspase-3 proteins in nude mice transplantation tumors during treatment with LFWGE (high-dose 2 g/kg/d; low-dose 1 g/kg/d) and 5-FU (25 mg/kg/d) (1, control group; 2, 5-FU group; 3, High-dose LFWGE, 2 g/kg/d; 4, Low-dose LFBE, 1 g/kg/d). *n*=6. Values represent the mean±SD. Different letters indicate a significant difference (*P*<0.05).

of human HT-29 cells in nude mice with anticarcinogenic properties. LFWGE can obviously inhibit HT-29 cell growth and apoptosis in nude mice.

The main function of CyclinD1 is to promote cell proliferation. CyclinD1 is the key protein regulating the G1 phase of the cell cycle. Many studies have shown that CyclinD1 is excessively expressed in a wide variety of tumor cells and leads to out-of-control and vicious cell proliferation[18]. Gómez-Alonso found that the anti-proliferative colon cancer effects induced by flavonols were in most cases preceded by a strong and significant reduction of cyclooxygenase-2 (COX-2) and CyclinD1 expression[19].

Apoptosis is a programmed cell death process that includes cell shrinkage, membrane foaming, chromatin condensation, nucleus division and ultimately the formation of apoptotic bodies[20]. Many intracellular molecules involve apoptosis, which plays an important role in maintaining the balance between tissues and organs. Apoptosis can be induced by either the body or external nutrients[21]. Studies have shown that inducing apoptosis has a dietary nutrition effect and does not affect normal cells[22]. The molecular mechanisms of apoptosis have been widely examined. Studies have found a variety of genes involved in apoptosis, such as the ced, Bcl-2, p53, STAT3 and Caspase gene families.

The Bcl-2 family plays a crucial role in the control of apoptosis. This family includes a number of proteins that have homologous amino acid sequences, including anti-apoptotic members such as Bcl-2 and Bcl-xL in addition to pro-apoptotic members including Bax and Bad. Bcl-2 blocks cell death, and Bax promotes programmed cell death. Bax and Bcl-2 can interact and form a complex regulatory network in the regulation of apoptosis. A high Bax expression increases cell apoptosis and a high Bcl-2 expression inhibits cell apoptosis[23].

In addition, apoptosis is a well-controlled process involving a programmed set of cellular events partially mediated by Caspases, which are the cysteine aspartate proteases involved in apoptosis that lead to the loss of cellular structure and function, phosphatidylserine exposure and ultimately cell death[24]. Caspase-3 is a key factor of apoptosis, and its activation is indicative of apoptosis entering an irreversible stage. Caspase-3 inactivates essential cellular substrates like DNA-repairing enzyme poly (ADP-ribose) polymerase-1, which is a

sterol regulatory element binding protein, by changing the structure of the substrate specificity or affecting a particular signal molecule to induce apoptosis[25].

In conclusion, this study clearly showed that LFWGE is a regulator of the transplantation tumor of human HT-29 cells in BALB/c nude mice and inhibits cell proliferation and apoptosis induction. Its mechanism may help increase expressions of Bax and the Caspase-3 gene and decrease expressions of Bcl-2 and the CyclinD1 gene. Based on the data, the development of fermented wheat germ extract with *Lactobacillus plantarum* dy-1 as the main component of anticarcinogenic function food laid the experimental foundation for this study. Future studies should examine the molecular mechanism of its anticarcinogenic effect in more detail.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

ZHANG Jia Yan designed the study, collected test data, interpreted the results, and wrote the manuscript. XIAO Xiang interpreted the results, and revised the manuscript. DONG Ying designed the study, interpreted the results, and wrote the manuscript. ZHOU Xing Hua collected test data and drafted the manuscript. WU Jing assisted in animal experiments and collected test data. All authors agreed to the final content.

REFERENCES

1. Kim JM, Kim JS, Yoo H, et al. Effects of black soybean [glycine max (l.) merr.] seed coats and its anthocyanidins on colonic inflammation and cell proliferation *in vitro* and *in vivo*. J Agric Food Chem, 2008; 56, 8427-33.

2. Zou YP, Chang S. Effect of black soybean extract on the suppression of the proliferation of human AGS gastric cancer cells via the induction of apoptosis. J Agric Food Chem, 2011; 59, 4597-5.

3. Liu RH. Potential synergy of phytochemicals in cancer prevention: mechanism of action. J Nutr, 2004; 134, 3479S-85S.

4. Judson PL, AlSawah E, Marchion DC, et al. Characterizing the efficacy of fermented wheat germ extract against ovarian cancer and defining the genomic basis of its activity. Int J Gynecol Cancer, 2012; 22, 960-7.

5. Duh P, Chen Z, Lee S, et al. Antiproliferative activity and apoptosis induction of eucalyptus citriodora resin and its major

bioactive compound in melanoma b16f10 cells. J Agric Food Chem, 2012; 60, 7866-72.

6. Brandolini A, Vidal AMH. Wheat germ: not only a by-product. Int J Food Sci Nutr, 2012; 63, 71-74.

7. Telekes A, Resetar A, Balint G, et al. Fermented wheat germ extract (avemar) inhibits adjuvant arthritis. Autoimmunity, Pt B: Novel Applications of Basic Research, 2007; 1110, 348-61.

8. Mueller T, Jordan K, Voigt W. Promising cytotoxic activity profile of fermented wheat germ extract [avemar(r)] in human cancer cell lines. J Exp Clin Canc Res, 2011; 30, 42-8.

9. Mueller T, Voigt W. Fermented wheat germ extract - nutritional supplement or anticancer drug? Nutrition Journal, 2011; 10, 89-94.

10. Rizzello CG, Nionelli L, Coda R, et al. Use of sourdough fermented wheat germ for enhancing the nutritional, texture and sensory characteristics of the white bread. Eur Food Res Technol, 2010; 230, 645-54.

11. Yoo JG, Kim MD. Production of 2-methoxy-1,4-benzoquinone (2-MBQ) and 2,6-dimethoxy-1,4-benzoquinone (2,6-DMBQ) from wheat germ using lactic acid bacteria and yeast. Food Eng Prog, 2010; 14, 292-8.

12. Rizzello CG, Mueller T, Coda R, et al. Synthesis of 2-methoxy benzoquinone and 2,6-dimethoxybenzoquinone by selected lactic acid bacteria during sourdough fermentation of wheat germ. Microbial Cell Factories, 2013; 12, 105-13.

13. Zhang JY, Xiao X, Dong Y, et al. Effect of Fermented Wheat Germ Extract with Lactobacillus plantarum dy-1 on HT-29 Cell Proliferation and Apoptosis. J Agric Food Chem, 2015; 63, 2449-57 .

14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 2001; 25, 402-8 .

15. Adachi Y, Taketani S, Oyaizu H, et al. Apoptosis of colorectal adenocarcinoma induced by 5-FU and/or IFN-gamma through caspase3 and caspase8. Int J Oncol, 1999; 15, 1191-7.

16. Wu XX, Kakehi Y, Mizutani Y, et al. Activation of caspase-3 in renal cell carcinoma cells by anthracyclines or 5-fluorouracil. Int J Oncol, 2001; 19, 19-24.

17. Comin-Anduix B, Boros LG, Marin S. Fermented wheat germ extract inhibits glycolysis/pentose cycle enzymes and induces apoptosis through poly(ADP-ribose) polymerase activation in Jurkat T-cell leukemia tumor cells. J Biol Chem, 2002; 277, 46408-14.

18. Zhong XY, Lin JM, Zhou JH, et al. Qianliening capsule treats benign prostatic hyperplasia (BPH) by down-regulating the expression of PCNA, CyclinD1 and CDK4. Afr J Biotechnol, 2012; 11, 7731-7.

19. Gómez-Alonso S, Collins VJ, Vauzour D, et al. Inhibition of colon adenocarcinoma cell proliferation by flavonols is linked to a G2/M cell cycle block and reduction in cyclin D1 expression. Food Chem, 2012; 130, 493-500.

20. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science, 1995; 267, 1456-62.

21. Naghma K, Afaq F, Mukhtar H. Apoptosis by dietary factors: the suicide solution for delaying cancer growth. Carcinogenesis, 2007; 28, 233-9.

22. Khan N, Afaq F, Saleem M, et al. Targeting multiple signaling pathways by green tea polyphenol(-)-epigallocatechin-3-gallate. Cancer Res, 2006; 66, 2500-5.

23. Youle RJ, Strasser A. The Bcl-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol, 2008; 9, 47-59.

24. Shi YG. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell, 2002; 9, 459-70.

25. Degterev A, Boyce M, Yuan JY. A decade of caspases. Oncogene, 2003; 22, 8543-67.