Nutrition 58 (2019) 100–109

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Grape seeds and skin induce tumor growth inhibition via G1-phase arrest and apoptosis in mice inoculated with Ehrlich ascites carcinoma

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ARTICLE INFO

Article History:
Received 28 February 2018
Received in revised form 17 May 2018
Accepted 24 June 2018

Keywords:
Grape seeds
Grape skin
Chemoprevention
Cell cycle
Apoptosis

ABSTRACT

Objectives: Chemoprevention or intervention of cancer by means of natural dietary components has shown great promise in controlling malignancy. This study was conducted to investigate the chemopreventive effects of grape seeds (GSE) combined with grape skin (GSK) in mice that were inoculated with Ehrlich ascites carcinoma, and to elucidate the underlying mechanisms.
Methods: GSE + GSK were mixed with the standard diet and supplemented to mice 14 d before Ehrlich ascites carcinoma cell inoculation and continued throughout the experiment. Tumor growth was monitored and cell cycle progression and apoptotic effect of GSE + GSK on tumor cells were evaluated.
Results: GSE + GSK intake prevented tumor development in 47% of the animals. Tumor volume and weight were markedly reduced by 93.9 % and 86.3 %, respectively, compared with tumor-bearing mice that were untreated with these agents. GSE + GSK treatment caused a marked increase in the percentage of apoptotic tumor cells as evaluated by flow cytometry and confirmed by histopathologic and electron microscopy examinations. GSE + GSK also caused significant cell cycle arrest at the G1 phase, activation of caspase-3, increase in p53 and Bax expression, and decrease in B-cell lymphoma 2 expression and B-cell lymphoma 2: Bax ratio in tumor cells. Furthermore, the induction of apoptosis and cell proliferation inhibition was indicated immunohistochemically as shown by modulating p53 and Ki67 expression.
Conclusions: The results of this study clearly showed that the combination of GSE and GSK represents a potent chemopreventive and anticancer agent in a mice model of Ehrlich carcinoma. The mechanisms that underlie the effects of these agents include cell cycle arrest, induction of apoptosis, and inhibition of cell proliferation. These findings suggest that GSE + GSK may represent a natural, novel, adjuvant therapeutic strategy for chemoprevention of the growth of solid tumors.

Introduction

Cancer chemoprevention represents a promising strategy to control the occurrence of cancer. Cancer chemopreventive drugs have been developed to prevent the growth of tumor cells in patients with cancer, but many of these drugs have shown to be toxic, such as amoxifen or raloxifene for breast cancer and nonsteroidal antiinflammatory colon cancer drugs. Therefore, naturally occurring compounds may offer promising alternative means for cancer chemoprevention. The ability of natural products to suppress carcinogenesis has attracted widespread attention as a means of cancer prevention and treatment [1–7]. Epidemiologic surveys and experimental studies have provided indications that dietary products such as fruits and vegetables play a noteworthy part in the regression of cancer [8,9] and that 30% of cancer morbidity and mortality can be prevented with proper adjustments of diets [10].

Grapes have been examined by many scientists for their ability to exert anticancer effects, which are believed to be related to a variety of their bioactive components [11]. With regular dietary intake, grape skin (GSK) and grape seeds (GSE) are more often discarded, but the consumption of the grape with its skin and seeds as part of a normal human diet may help maintain great wellbeing.

Grapes contain large amounts of phytochemicals including anthocyanins, catechins, resveratrol, phenolic acids, and procyanidins [12]. Grape skin is enriched with resveratrol and catechin, and grape seeds contain concentrated procyanidins [13]. Resveratrol has been shown to possess preventive and treatment effects against several types of malignancies in experimental animal

Conflicts of interest: The authors declare that no competing interest exists.

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https://doi.org/10.1016/j.nut.2018.06.018
0899-9007/

studies and against several human diseases including cancer [14,15]. Resveratrol exerts antiproliferation effects in various human tumor cell lines [16] through multiple cell signaling pathways such as the arrest of cell cycle, inhibition of tumor cell proliferation, and induction of apoptosis [17]. Similarly, grape seed proanthocyanidins are promising phytochemicals that, to a great extent, are responsible for the spectrum of biological activities demonstrated by this extract and exert antioxidant and antiinflammatory properties, which have shown minimal toxic effects in laboratory animals [18–21].

Recently, grape seeds have been shown to suppress proliferation and promote apoptosis in the liver of rats treated with diethylnitrosamine and 2-acetyl aminofuorene [22] as well as induce apoptosis in human colorectal carcinoma cell lines through a mechanism that involves a reduction in mitochondrial membrane potential [23]. Other studies have shown that grape seed extract enhances the uptake of oxaliplatin chemotherapy into different cancer cell lines by modulating the cell transport system [24].

To date, the majority of studies that have examined the anticancer efficacy of grape were carried out in vitro using individual grape ingredients against cancer cell lines. In addition, the number of clinical trials that have explored the anticancer activity of grape in humans is limited but increasing [11]. Therefore, more investigations are expected to better assess the ability of grapes to exert anticancer activity on animal bearing tumor.

We hypothesize that combining GSE and GSK with a standard diet will exhibit a great chemopreventive effect when administered to mice that bear Ehrlich ascites carcinoma (EAC) cells. Our aim is to determine the underlying mechanism of tumor inhibitory effects of GSE and GSK by studying the mitochondrial pathway-induced apoptosis and cell cycle progression.

Methods

Experimental animals

A total of 50 adult female Swiss albino mice (ages 2-3 mo) that weighed 18 to 21 gm were used in the present study. Animals were housed in specific pathogen-free plastic cages and maintained under controlled conditions of humidity (55%), temperature (25 ± 1°C), and in a diurnal environment (12 h light/dark cycle). The mice were kept for 1 wk before the experiment for acclimatization and fed a standard rodent diet composed of 20% casein, 15% corn oil, 55% corn starch, 5% salt mixture, 5% vitaminized starch (Egyptian Company of Oil and Soap, Kafr Elzayat, Egypt), and permitted unlimited access to water. The experiment was performed in compliance with the guidelines of the Guide for the Care and Use of Laboratory Animals. All experiments were approved by the University of Mansoura Animal Care Committee.

Ehrlich ascites carcinoma cell line and tumor transplantation

EAC was supplied by the National Cancer Institute, Cairo University in Egypt, and maintained by weekly intraperitoneal transplantation of 2.5×10^6 cells in female mice. EAC viability was assessed with the Trypan blue-dye exclusion method. EAC cells (2.5×10^6) were transplanted subcutaneously (SC) into the back of the mice.

Preparation of grape seeds and skin

Vitis vinifera GSE + GSK were detached from the pulps manually, dried, and ground to powder, and then mixed homogeneously with the standard diet at concentrations of 10% (w/w) [25].

Experimental design

Mice were grouped as follows: 10 untreated control mice (group I [C]), 10 mice that were fed the regime diet (group II [GSE + GSK]), 15 mice that were injected SC in the back with EAC cells (2.5×10^6) for solid tumor induction (group III [EAC]), and 15 mice that were fed the regime diet for 14 d and then each mouse was injected SC in the back with EAC cells (2.5×10^6) for solid tumor induction and the diet regime maintained for the duration of the study (group IV [EAC + GSE + GSK]). The animals were monitored for feeding/drinking and life activity habits including change in animal body weight and mortality for the course of the experiment. The detection of palpable tumors and tumor growth was monitored regularly by measuring changes in tumor volume (TV/mm^3) during the experimental time course. A Vernier caliper was used to collect information on tumor size data that was plugged into the following formula: TV (mm^3) = 0.52 AB2, where A is the minor axis and B is the major axis. On day 30 of tumor cell inoculation, the mice were sacrificed and the solid tumor mass was removed and used for tumor weight (TW) determination and different investigations.

Histopathologic evaluation

Histopathology changes in the tumor tissues of groups III and IV were evaluated using a light microscope. Tumor tissues were fixed in 10% neutral buffered formalin before being processed for paraffin sections (4 μm thickness) and stained with hematoxylin and eosin.

Immunohistochemical evaluation of p53 and Ki67 expression

Sections of tumor tissue were cut onto positive-charged slides and incubated with monoclonal antip53 and antiki67 antibodies (1: 500) for 2 h at 4°C. The sections were applied with the secondary antibody and the avidin-biotin complex. Staining with 3,3′-diaminobenzidine was used as a chromogen and Mayer's hematoxylin as a counterstain to the sections. Any sections with <5% positive cells were recorded as negative [26].

Transmission electron microscopic studies

The tumor cells of groups III and IV were immediately harvested and fixed in 4 F1 G in phosphate buffer (pH 7.2) at 4°C and postfixed for 3 h in 1% cold osmium tetroxide in phosphate buffer at pH 7.2. The dehydration of the specimens was carried out in graded ethanol before the sections were embedded in Epson-Araldite resin. Uranyl acetate and lead citrate were used to stain the ultrathin sections [27], which were then examined with a JEOL Electron Microscope (Japan) that operates at 60 kV.

Cell preparation for flow cytometry

Tumor tissue samples were prepared with a manual disaggregation procedure. In short, we added a few drops of Roswell Park Memorial Institute medium to the tissues and then achieved complete tissue disaggregation through mincing. Suspended cells were processed with a 50 μm pore-size mesh to remove residues and then centrifuged at 1000 rpm for 10 min. The cells were resuspended in phosphate-buffered saline (PBS), counted, washed with PBS, and subsequently centrifuged at 1500 rpm for 5 min. The cell suspension was fixed in 70% ice-cold ethanol in PBS, and stored at −20°C until use [28].

Flow cytometry analysis of cell cycle progression and apoptosis

To determine cell cycle phase distribution and the percentage of apoptotic cells, the EAC cell suspensions of tumor-bearing mice in the presence or absence of GSE + GSK treatment were centrifuged, and the cell pellets were resuspended in 1 mL of propidium iodide (PI) solution (Sigma, Missouri; 25 μg/mL PI [wt/vol], 0.1 Mm ethylenediaminetetraacetic acid, and 10 μg g/mL RNase [wt/vol] in PBS) for 30 min in the dark. DNA contents were measured to analyze apoptotic cell death [29] using a FACS caliber flow cytometer (Becton-Dickinson, Sunnyvale, CA) that was equipped with a compact, air-cooled, low-power, 15 m watt argon iron laser beam (488 nm). We scanned nuclei at a rate of 120/s, and the number of evaluated nuclei per specimen averaged 20 000. The computer program MoDFit was used to obtain cell cycle phase distribution of nuclear DNA for a mathematical analysis [30].

Quantitative determination of apoptotic cell death using Annexin V/PI double staining

Annexin-V is a protein that binds to phosphatidylserine (ps) residues that are exposed on cell surfaces of apoptotic but not normal cells. The ps groups are exposed to the exterior of the cell membrane during apoptosis, and the binding of ps to Annexin-V has been shown to be a biochemical marker of apoptosis. The induction of apoptosis that is caused by GSE + GSK in tumor cells was quantitatively determined by flow cytometry using the Annexin-V-conjugated Alexafluor 488 Apoptosis Detection Kit in accordance with the manufacturer's instructions (Biosciences, Sose, CA).

Flow cytometry analysis of p53, Bax, and B-cell lymphoma 2 expression

To determine the expression of the pro-apoptotic protein p53, Bax or, antiapoptotic protein Bcl-2, tumor cells (1×10^6) from mice

in the presence or absence of GSE + GSK treatment were incubated either with mouse antip53, anti-Bax, or anti-Bcl-2 for 1 h at room temperature, and then with fluorescein isothiocyanate (FITC)-conjugated goat antirabbit antibody. The cells were washed thoroughly and analyzed on a flow cytometer (Becton Dickinson, CA). We acquired a total of 20 000 cells with CellQuest software. The histogram plot of FITC (x axis) versus counts (y axis) has been shown in the intensity of logarithmic fluorescence.

Quantification of caspase-3 activity by flow cytometry

The ab65613 caspase- 3 (active) FITC staining kit (Abcam) was employed to determine the activity of caspase-3. The assay uses as a marker the caspase- 3 inhibitor, DEVD-FMK, that is conjugated to FITC (FITC-DEVD-FMK). FITC-DEVD-FMK is non-toxic, cell permeable, and binds irreversibly with activated caspase-3 in apoptotic cells. The FITC label uses flow cytometry directly to detect activated caspase -3 in apoptotic cells. We mixed cells with FITC-DEVD-FMK and incubated them for 0.5 to 1 h in a 37°C incubator with 5% carbon dioxide. The cells were centrifuged at 3000 rpm for 5 min and supernatant was removed. The cells were resuspended in 300 μl of wash buffer, and the samples were stored on ice until analyzed by flow cytometry via the FL-1 channel.

Statistical analysis

The values are reported as mean ± standard error of measurement (SEM), and the data were analyzed using one-way analysis of variance, followed by post hoc tests for multiple comparisons or Student *t* test where appropriate. A *P*-value of <0.05 was considered statistically significant.

Results

Body weight

Change in body weight (BW)/gm in the different groups was recorded weekly throughout the length of the experiment. At day 30 of posttumor cells inoculation, normal untreated mice (C group) showed a BW gain of 3.3 gm (+13.9% of initial BW), and the control mice that were treated with GSE + GSK had a gain of 2.7 gm (+11.1% of initial BW). Untreated mice-bearing solid tumor (EAC group) showed a body weight loss of 1.1 gm (−3.9% of initial BW), but the EAC + GSE + GSK group recorded 1.9 gm BW gain (+7.9% of initial BW; Fig. 1).

Tumor growth

Tumor incidence

Palpable tumors were observed at day 9 of tumor cells inoculation in 15 of 15 mice of the untreated EAC group (100% malignancy). In contrast, the mice treated with GSK + GSE showed marked inhibition in the growth of the tumors for 8 of 15 mice (53% malignancy), and the remaining 7 of 15 mice (47%) were tumor free. Among the 8 of 15 mice that showed inhibition, complete tumor regression was recorded in two mice on days 22 and 26 of tumor cells inoculation (2 of 8 mice; data not shown).

Tumor volume

The measurement of tumor volume (TV) at regular intervals throughout the experimental period indicated that GSE + GSK dramatically suppressed tumor growth. TV/mm^3 at the end of the experiment reached 1082.1 ± 71.7 mm^3 in untreated, tumor-bearing mice (EAC group), but was only 66.2 ± 6.9 mm^3 for mice treated with GSE + GSK. This represents a tumor regression by 93.9% ($P < 0.01$; Fig. 2A).

Tumor weight

When the experiment ended, we dissected the tumor mass from each mouse and recorded wet weight. Treatment with GSE + GSK caused a marked reduction in tumor weight (TW) by 86.3% ($P < 0.01$) compared with the control group of untreated EAC mice (Fig. 2B). The mean value of TW was 0.96 ± 0.2 gm and 0.13 ± 0.1 gm for the EAC group and mice treated with GSE + GSK, respectively. These results indicate that GSE + GSK can provide significant cancer chemoprevention in mice.

Mortality

Mortality incidence was recorded throughout the experimental period. Animal mortality rate reached 26.7% (4 of 15 mice) for the control group of untreated EAC mice, and no mortality was recorded for the other groups (data not shown).

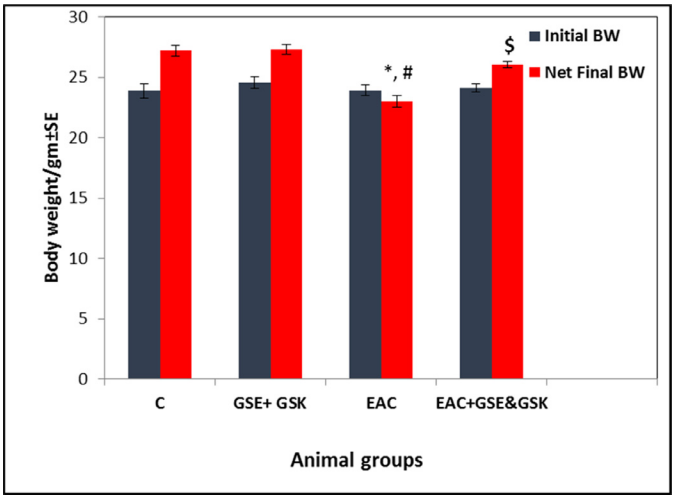

Fig. 1. Effect of grape seeds (GSE) and grape skin (GSK) intake on body weight (BW)/gm. Each value represents the mean ± standard error of measurements. Number of mice/group for initial and net final BW: Control group (10 and 10), GSE + GSK group (10 and 10), Ehrlich ascites carcinoma (EAC; 15 and 11), EAC + GSE + GSK (15 and 15). Net final BW = last BW − tumor weight. *$P < 0.05$ compared with control group. #$P < 0.05$ compared with GSE + GSK group. $$P < 0.05$ compared with EAC group.

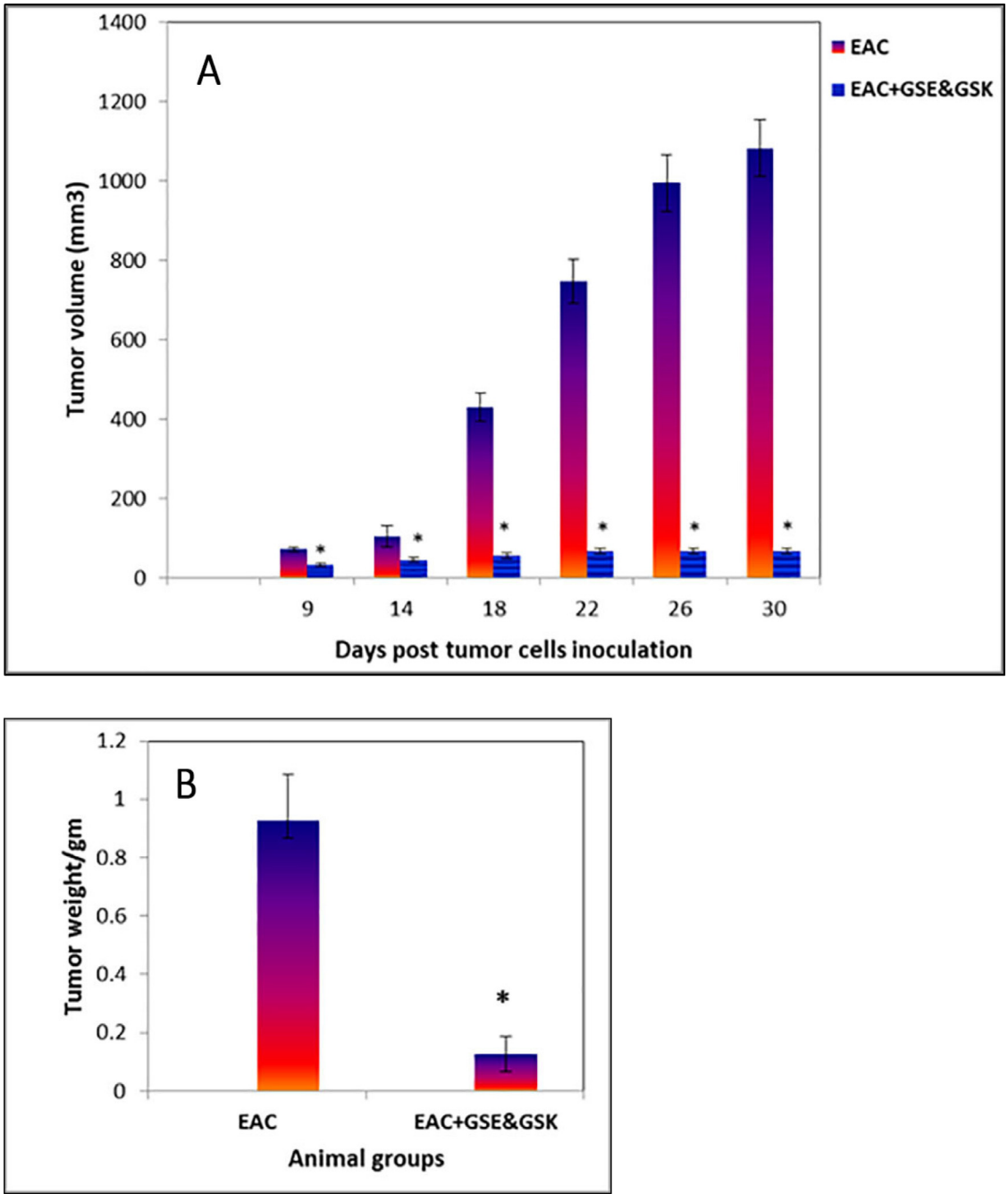

Fig. 2. Effect of grape seeds (GSE) and grape skin (GSK) intake on tumor volume (TV) and tumor weight (TW). (A) TV change: TV/mm^3 was recorded on a regular basis. Palpable tumors were observed at day 9 after tumor cell inoculation in 15 of 15 mice of the untreated Ehrlich ascites carcinoma (EAC) group (100% malignancy) and 8 of 15 mice in EAC + GSE + GSK group (53% malignancy). Complete tumor regression was recorded in two mice on days 22 and 26 of tumor cell inoculation (2 of 8 mice). TV/mm^3 was compared with the corresponding untreated tumor-bearing mice. Each value represents the mean ± standard error of measurements. (B) TW/gm was determined at the end of the experiment. Each value represents the mean ± standard error of measurements of 11 mice of untreated EAC and six mice of the EAC group treated with GSE + GSK. * $P <$ 0.01 compared with untreated EAC group.

Histopathological examination

The untreated EAC group reveals tumor tissue that infiltrated the muscle layer and formed solid sheets of undifferentiated tumor cells, separated by wide spaces of coagulative necrosis. The tumor cells show high grades of anaplasia, pleomorphism, and abnormal mitotic figure (Figs. 3A and B). Mice treated with GSE + GSK revealed marked clusters of individual apoptotic tumor cells that appeared shrunken with deeply eosinophilic cytoplasm and pyknotic nuclei (Figs. 3C and D).

Electron microscopy examination

EAC cells of control (untreated) mice exhibited nuclear heterochromatin with oval nuclei (Fig. 4A) and tumor cells that showed oval nuclei, nuclear envelope, nuclear pore, heterochromatin, numerous

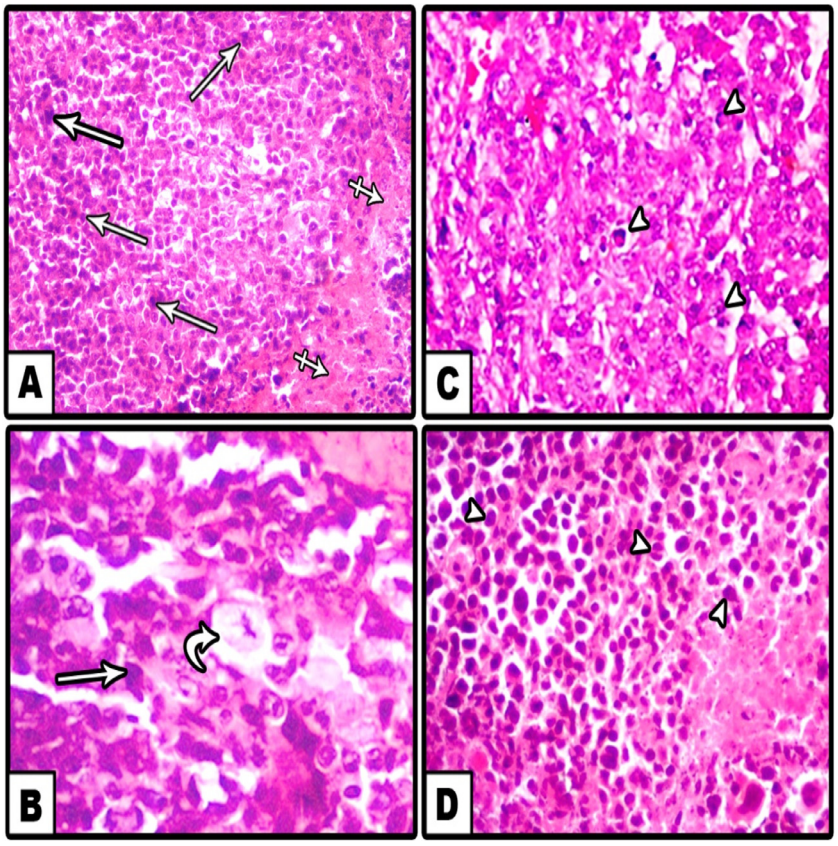

Fig. 3. Histopathology of the tumor tissue (Ehrlich ascites carcinoma [EAC]) with or without grape seed (GSE) and grape skin (GSK) treatment. (A) and (B) Left panel: represents control untreated EAC mice with (A) the tumor tissue infiltrating the muscle layer and forming solid sheets of undifferentiated tumor cells (arrows), separated by wide areas of coagulative necrosis (crossed arrow), and (B) the tumor cells showing high grade of anaplasia, pleomorphism, and abnormal mitotic figure (curved arrow, hematoxylin and eosin × 200, × 400 respectively). (C) and (D) Right panel: represents GSE + GSK treated tumor. (C) Marked clusters of individual apoptotic tumor cells (arrow heads). (D) Apoptotic tumor cells appear shrunken with deeply eosinophilic cytoplasm and pyknotic nuclei (arrow heads, hematoxylin and eosin × 200, × 400, respectively).

ribosomes, and rough endoplasmic reticulum (Figs. 4B and C). Morphologic changes that are typical of apoptosis were observed in EAC cells of mice treated with GSE + GSK. This group of mice showed degenerated and apoptotic cancer cells (Fig. 4D), apoptotic nuclei with oval and crescent chromatin condensation and destructed mitochondria (Fig. 4E), with the appearance of apoptotic bodies that revealed the presence of nuclear fragmentation (Fig. 4F).

Flow cytometry analysis of cell cycle progression and apoptosis in tumor cells

An analysis of cell cycle progression and apoptotic death was performed at day 30 posttumor cells inoculation using flow cytometry of PI-stained cells. Figures 5A and B show that treatment with GSE + GSK resulted in cell cycle arrest at G0/G1 by demonstrating a highly significant increased cell population at the G1 phase (31.1%; $P < 0.001$), followed by reduced numbers at the S and G2 phases. GSE + GSK intake caused significant accumulation of cells in the G1 phase and decreased the S phase cell population by −72.4%. Overall, the observed cell cycle arrest (predominantly in the G1 phase) by GSE + GSK possibly accounts for its cell growth inhibitory effect in EAC cancer cells.

The levels of apoptosis as detected by the PI-staining of DNA fragmentation and represented by sub-G1 peak are shown in Figures 5A and C. The hypodiploid cells in the sub-G1 phase of the EAC cells of the mice in the untreated EAC group showed 12.8 ± 1.1% apoptotic cells. On the other hand, the tumor cells of the mice in the GSE + GSK treated group showed a highly significant increase in the percentage of apoptotic cells (55.3 ± 4.6%; $P < 0.01$). This represents a 4.3-fold increases in the percentage of apoptotic tumor cells.

Flow cytometry analysis of apoptosis as determined by AnnexinV/PI

A quantitative analysis of apoptosis in tumor cells was performed using Annexin V/ PI double stain at day 30-posttumor cell inoculation. As shown in Figure 6, the pretreatment of GSE + GSK resulted in an increased percentage of early apoptotic cells by 421.4% ($P < 0.01$) and an increased percentage of the late apoptotic population by 384.9% ($P < 0.01$) compared with the untreated EAC group. The percentage of total apoptotic cells was 393.0% ($P < 0.01$) of the untreated control value, which indicates great apoptotic effect of GSE + GSK treatment.

Flowcytometry analysis of the expression of apoptotic regulators in tumor cells

To further elucidate the mechanism of GSE + GSK-induced apoptosis in tumor cells, the percentage of protein levels of apoptosis-linked gene products p53, Bax, Bcl-2, and caspase-3 activity were measured using flow cytometry (Table 1). Compared with the tumor cells of mice in the untreated group, the supplementation of GSE + GSK increased p53 and Bax expression by 333.0% and 113.3%, respectively ($P < 0.01$), which represents 4.3- and 2.1-fold increase, respectively, compared with the untreated EAC group, and resulted in 91% inhibition of Bcl-2 expression ($P < 0.01$). This

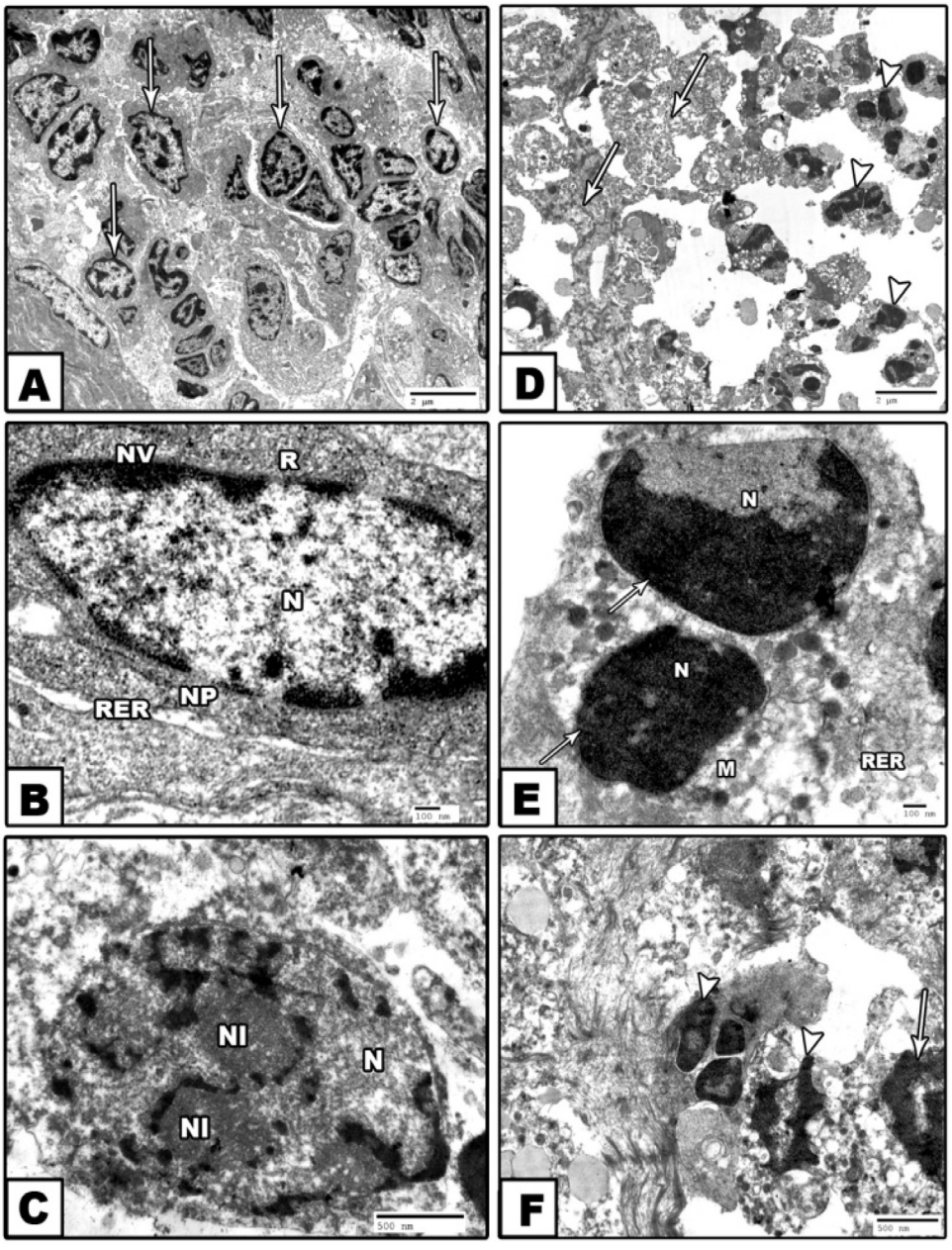

Fig. 4. Electron micrograph of Ehrlich ascites carcinoma (EAC) cells of mice with or without grape seed (GSE) and grape skin (GSK) treatment. (A), (B), and (C) Left panel: EAC cells of untreated mice. (A) Numerous active EAC cells (× 1000). (B) Tumor cells showing oval nuclei (N), nuclear envelope (NV), nuclear pore (NP), heterochromatin, numerous ribosomes (R), and rough endoplasmic reticulum (RER) (× 7500). (C) Tumor cells with two nucleoli (NI) and heterochromatin, (× 5000). (D), (E), and (F) Right panel: morphologic changes typical of apoptosis were observed in EAC cells of mice treated with GSE + GSK. (D) EAC cells of GSE + GSK treated mice showing few degenerated (arrow) and apoptotic (arrow head) cancer cells (× 1000). (E) Two apoptotic nuclei (arrow) with oval and crescent chromatin condensation (RER) and destructed mitochondria (M; × 7500). (F) Apoptotic cancer cell with chromatin condensation (arrow) and chromatin fragmentation forming apoptotic bodies (arrow head; × 4000).

was accompanied with a marked decrease of 96.1% in the ratio of Bcl-2:Bax protein expression with respect to the tumor cells of the mice in the untreated EAC group. GSE + GSK intake markedly induced the activation of Caspase-3 in EAC cells. Activity was significantly increased by 118.9% ($P < 0.01$), which is 2.2-fold higher than the EAC cells of untreated control mice.

Immunohistochemical demonstration of p53 and ki67 expression in tumor cells

The effect of GSE + GSK treatment on the expression of the proapoptotic p53 and the cell proliferation marker Ki67 in tumor tissues was determined immunohistochemically. Control untreated tumor tissues showed a low expression of p53 protein (Fig. 7A), but an enhanced expression of p53 protein was observed in mice that were treated with GSE + GSK (Fig. 7B), which confirms the generation of apoptosis in tumor cells in mice. The expression of Ki67 protein in tumor sections was also examined and showed strong nuclear immunoreactivity in untreated animals (Fig. 7C), but showed negative nuclear immunostaining in animals treated with GSE + GSK (Fig. 7D). Therefore, our results suggest that GSE + GSK treatment inhibits the proliferation of tumor cells through cell cycle arrest and by activating apoptosis in vivo.

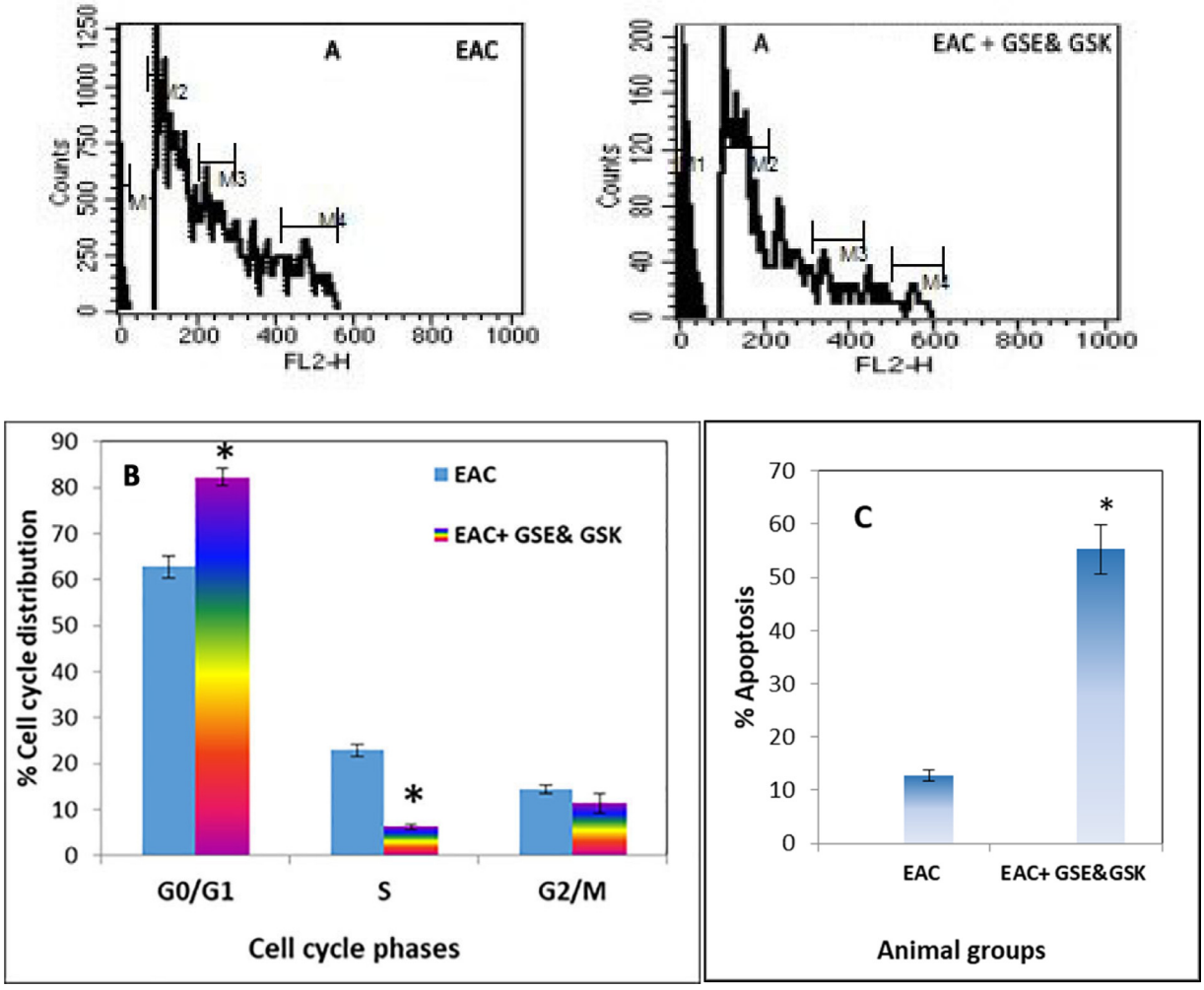

Fig. 5. Effect of grape seed (GSE) and grape skin (GSK) intake on cell cycle progression of Ehrlich ascites carcinoma (EAC) cells harvested from mice tumor at day 30 of tumor cell inoculation. Treatment of GSE + GSK caused marked changes of the cell cycle profile. (A) Representative histograms of untreated EAC group (left) or mice treated with GSE + GSK (right) of flow cytometry analysis of propidium iodide-stained cells showed M1 (sub-G1) peak, which is a characteristic of nuclear apoptosis; M2 (G0/G1); M3(S); and M4 (G2/M) phases. (B) Quantitative cell cycle distribution: GSE+ GSK treatment resulted in cell cycle arrest at G0/G1 ($^*P < 0.01$) with reduced numbers at the S and G2 phases. (C) Histogram represents the percentage of apoptosis. The content of hypoploid DNA was 12.8% for the untreated group versus 55.3% for the mice treated with GSE + GSK. Data were expressed as means ± standard error of measurements of 6 mice/group.: $^*P < 0.01$.

Discussion

One promising strategy to control the occurrence of cancer is through cancer chemoprevention. However, the toxicity of contemporary cancer chemopreventive drugs are of great concern. Therefore, naturally occurring compounds may offer promising alternative means for cancer chemoprevention.

The present study examines the use of the grape *Vitis vinifera* as a chemopreventive agent. The experimental approach was designed to investigate the possible use of crude GSE + GSK as an effective chemopreventive agent in EAC mice models. The experimental approach can be summarized as an examination of GSE + GSK as a cancer chemopreventive agent, and of the underlying mechanisms of GSE + GSK as chemopreventive agents.

The present findings demonstrate that treatment with GSE + GSK caused profound suppression of tumor growth in mice via mechanisms that involve cell cycle arrest, induction of apoptosis, and inhibition of cell proliferation. The results also show that apoptosis was mediated by the modulation of apoptotic regulators in tumor cells. In the present study, we analyzed the antitumor effects of GSE + GSK in vivo using mice inoculated with EAC. Ehrlich carcinoma is an excellent choice for this study because of its high transplantable capability, 100% malignancy, no regression, and quick proliferation. Ehrlich carcinoma is also short-lived and does not have a tumor-specific antigen [31].

Treatment with GSE + GSK resulted in significant inhibition of tumor growth as manifested by a dramatic decrease in tumor incidence, volume, and weight. Histopathologic studies showed that GSE + GSK resulted in marked drop in tumor-cell density, increased apoptotic cancer cells, and an expansion of the degenerative areas. These findings were confirmed by electron microscopic studies that showed features of degeneration and apoptotic cancer cells. Earlier studies have shown that the bioactive components of GSE and GSK are potent anticancer agents. For example, exposure to resveratrol caused inhibition in tumor development in animals bearing tumors and in cancer cell lines via apoptosis induction in cancer cells and proliferation arrest [32–35]. Additionally, further studies revealed the cancer chemopreventive effects of the phenolic compounds of grapes against breast carcinogenesis and cancer progression in rodent models [36–39]. Furthermore, grape proanthocyanidin extracts have been shown to minimize the occurrence of carcinogen-induced animal mammary and skin tumors, as well

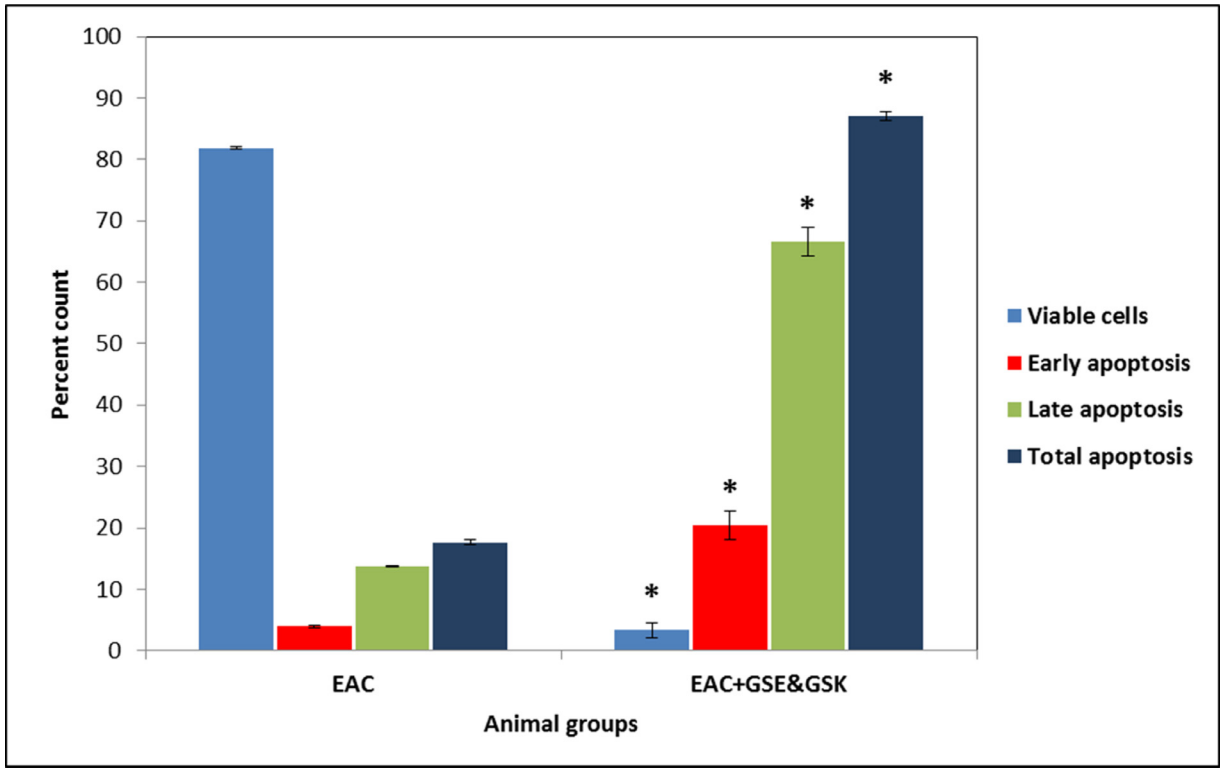

Fig. 6. Effect of dietary grape seed (GSE) and grape skin (GSK) intake on apoptosis in Ehrlich ascites carcinoma-bearing mice as detected by Annexin V/propidium iodide and analyzed by flow cytometry at day 30 posttumor cell inoculation. Data represented as means ± standard error of measurements of 6 mice/group. *:$P < 0.01$ compared with the untreated Ehrlich ascites carcinoma group.

Table 1
Effect of GSE + GSK intake on the percentage of apoptotic regulators p53, Bax, Bcl-2 expression, Bcl-2:Bax ratio, and caspase-3 activity in EAC tumor cells as determined by flow cytometry at day 30 posttumor cells inoculation

Group	% p53 expression	% Bax expression	% Bcl-2 expression	Bcl-2:Bax ratio	% caspase-3 activity
EAC	15.3 ± 0.6	25.2 ± 1.4	75.0 ± 2.9	3.0 ± 0.1	22.9 ± 1.8
EAC + GSE + GSK	66.4 ± 1.3*	53.6 ± 5.3*	6.24 ± 0.5*	0.116 ± 0.01*	50.1 ± 3.6*
% change of EAC	(+333.0%)	(+113.3%)	(−91.7%)	(−96.1%)	(+118.9%)

Bcl-2, B-cell lymphoma 2; EAC, Ehrlich ascites carcinoma, GSE, grape seeds, GSK, grape skin.
Data represent mean ± standard error of measurements of 6 mice/group and percentage of change of the EAC group.
*$P < 0.01$ compared with untreated EAC group.

as prevent the growth of human cancer cells of various phenotypes in vitro and in vivo [40–43]. GSE and GSK are the primary distributors of grape phenolic compounds [44], but resveratrol is present at high levels in GSK and procyanidins are concentrated in GSE [13].

An effective strategy to inhibit tumor growth has been shown to control cell cycle progression in cancer cells. In most of the common malignancies, cell cycle regulators are often deregulated, as revealed by molecular analyses of human cancers [45,46]. Our results show that the efficacy of GSE + GSK in the induction of tumor regression involves cell cycle arrest. Treatment with GSE + GSK resulted in a marked elevation in the sub-G0/G1 population (hypodiploid DNA content), which is a characteristic mark of apoptosis.

Moreover, our data on cell cycle distribution indicate that GSE + GSK induced a remarkable elevation in cell number in the G0/G1 phase with a parallel decrease in the other phases. Earlier studies have shown that resveratrol has the ability to cause disruption of the G1/S transition of the cell cycle in various cancer cell lines [35,47,48].

The appearance of the sub-G1 population was with characteristic morphologic changes in cytoplasm as well as with chromatin condensation and nuclear disentegration, which are common features of apoptosis and detected using light and electron microscopy. Proanthocyanidin has been shown to promote cell cycle arrest and apoptosis in metastatic breast carcinoma cells, as well as suppress tumor xenograft growth [49]. Additionally, a quantitative analysis of apoptosis was performed using Annexin V/PI double staining and demonstrated that GSE + GSK markedly increased the percentage of early and late apoptotic cells compared the untreated EAC group.

The translocation of phosphatidyl serine indicates extensive damage to cell membrane. This corroborates previous reports that demonstrated a significant elevation in the percentage of early and late apoptosis, together with significant inhibition in the viable cell count after treating different cancer cell lines with grape seed extracts [49,50].

One possible mechanism to inhibit cancer development is to induce apoptosis and, indeed, many anticancer agents rely on this mechanism to suppress or block the carcinogenic process. The

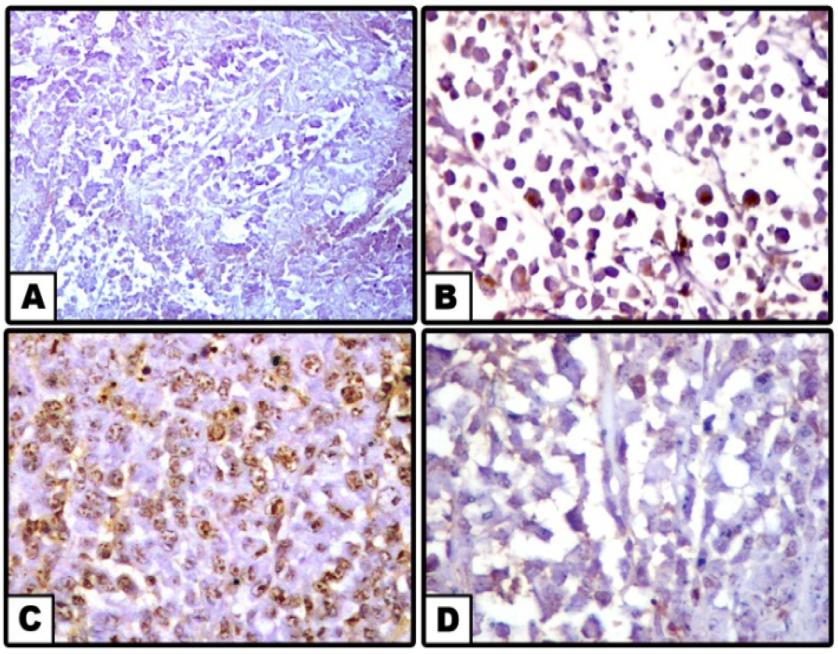

Fig. 7. Immunostaining of P53 and Ki67 in tumor tissue of mice untreated or treated with grape seeds (GSE) and grape skin (GSK). Sections of tumor tissue were cut onto positive-charged slides and incubated with monoclonal anti-p53 antibody as well as with antiki67 antibody (1:500) for 2 h at 4°C. The secondary antibody and avidin-biotin complex were applied to sections, 3,3′-diaminobenzidine was used as a chromogen, and sections were counterstained with Mayer's hematoxylin. (A) Negative nuclear immunostaining for p53 in the untreated Ehrlich ascites carcinoma group. (B) Positive nuclear immunostaining for p53 in the GSE + GSK treated group. (C) Strong nuclear immunoreactivity for Ki67 in untreated tumor tissue. (D) Negative nuclear immunostaining for Ki67 in GSE + GSK treated tumor tissue (× 400).

results of this study show that the effectiveness of dietary GSE + GSK in the induction of tumor regression was associated with an induction in cancer cell apoptosis, as seen in the significant changes in the expression of apoptotic regulators (activation of caspase-3, upregulation in the expression of proapoptotic proteins p53 and Bax, downregulation in the expression of the antiapoptotic protein Bcl-2, and decreased Bcl2: Bax ratio).

Furthermore, the induction of apoptosis and inhibition of cell proliferation in tumor cells with dietary GSE + GSK intake was observed in tumor tissues by modulating p53 and Ki67 immunoreactivity. Whether or not a cell begins the process of apoptosis depends in part on the balance of proteins that mediate cell death and growth arrest. Earlier studies have shown that one of these proteins, p53, promotes apoptosis [51], and that the Bax protein pathway mediates this process. The permeability of the mitochondrial membrane can be enhanced by p53 transcriptional activation of pro-apoptotic Bax, which results in a release of cytochrome c and in turn acts through a cascade of events to the activation of caspase-3 (one of the key mediators of p53-induced apoptosis) [1,52,53]. In our study, treatment of GSE + GSK was found to result in a decrease Bcl-2:Bax ratio and an activation of caspase-3 in cancer cells, which has been shown to be essential for DNA fragmentation and chromatin condensation [54] as evidenced by the present light and electron microscopy observations.

Conclusions

The present data reveal that the combination of dietary GSE + GSK supplements effectively inhibited tumor growth via mechanisms that involve cell cycle arrest and apoptosis. Our in vivo study provides evidence that GSE + GSK may possess great potential for natural chemoprevention of the growth of solid tumors. In the future, studies on the cancer-fighting properties of each grape component and the initiation of clinical trials in humans with different types of malignancies will be of interest.

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