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Fresh Apples Suppress Mammary Carcinogenesis and Proliferative Activity and Induce Apoptosis in Mammary Tumors of the Sprague−Dawley Rat

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Whole apple extracts possess potent antioxidant activity and antiproliferative activity against cancer cells in vitro. The objectives of this study were to determine the anticancer activity of apple extracts in a rat mammary cancer model induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) in vivo and to determine if apple extracts inhibited cell proliferation and affected apoptosis in mammary cancer tissues in vivo. Rats were given the whole apple extracts (0, 3.3, 10.0, or 20.0 g/kg of body weight) by gavage starting 2 weeks prior to DMBA administration and continuing for 24 weeks. Rats treated with DMBA (positive control) developed mammary tumors with 71.4% tumor incidence during the 24-week study. No tumors were detected in the negative control group untreated with DMBA. A dose-dependent inhibition of mammary carcinogenesis by apple extracts was observed ($P < 0.01$). Tumor multiplicity decreased with increasing apple extracts. Histopathological evaluations of tumors were performed. The proportions of adenocarcinoma masses decreased with increasing apple extracts. The expression of proliferating cell nuclear antigen (PCNA), cyclin D1, and Bcl-2 decreased, and Bax expression and apoptosis increased with increasing apple extracts. These results demonstrate the potent capacity of fresh apples to suppress DMBA-initiated mammary cancers in rats.

KEYWORDS: Apple; mammary carcinogenesis; cell proliferation; apoptosis; breast cancer; diet

INTRODUCTION

Breast cancer is the most frequently diagnosed invasive cancer and the second leading cause of cancer deaths in women in the United States. It was estimated that approximately 1,780,480 new cases would have been diagnosed and more than 70,880 women would have died from breast cancer in the United States alone in 2007 (*1*). There is no certain means of preventing breast cancer. All women can limit their risk factors for breast cancer, and women at high risk for the disease may be candidates for medical and surgical preventive measures. Currently, management options for women to decrease their breast cancer risk include intense breast cancer surveillance, surgical prophylaxis (i.e., prophylactic mastectomy), and preventive therapy with tamoxifen. Surgery provides the greatest risk reduction, but because of its severe physiological and psychological consequences, it is considered in only very high-risk cases (*2*, *3*). The nonsteroidal antiestrogen medicine tamoxifen has been reported to be effective in only 40% of breast cancer patients (*4*). Thus, recognition of the limitations of current diagnostic, surgical, and therapeutic approaches to breast cancer has resulted in a new focus on breast cancer chemoprevention.

It has been reported that >30% of human cancers could be prevented by an alternative strategy of appropriate dietary modification (*5*, *6*).

Epidemiological studies, including case-control and cohort studies, have consistently shown that regular consumption of fruits and vegetables is associated with reduced risk of developing cancer and other chronic diseases (*7*). It has been suggested that the health benefits of fruits and vegetables are attributed to the complex mixture of the additive and synergistic effects of phytochemicals present in whole foods, rather than to a single compound (*8*, *9*). In a meta-analysis of 16 case-control studies and 3 cohort studies, a 25% lower breast cancer risk was found for high versus low consumption of vegetables and a 6% lower risk for high versus low consumption of fruits (*10*). A recent epidemiological study including 2569 breast cancer patients showed that apple consumption was linked to a lower risk of breast cancer (*11*). However, some cohort studies do not show the same protective effect (*12*, *13*). Thus, it is still unclear

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whether specific fruits, vegetables, or compounds are responsible for this relationship.

Apples are a very significant part of the diet and are one of the best sources of antioxidant phenolic compounds in the Western world (*14*). In our previous studies, apple extracts have been shown to have potent antioxidant activity and antiproliferative activity against colon, liver, and breast cancer cells in vitro in a dose-dependent manner (*15*−*18*). Apples were ranked second for total phenolic content and had the second highest total antioxidant activity among 21 fruits and vegetables commonly consumed in the United States (*16*, *19*). In a previous paper, we reported apple extracts inhibited 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary cancer in rats in a dose-dependent manner (*20*). Recently, we reported apple extracts had activity inhibiting NFkB activation in human breast cancer MCF-7 cells (*18*). The objectives of the present study were (1) to determine anticancer activity of apple phytochemical extracts in a DMBA-induced rat mammary cancer model in vivo, (2) to determine the ability of apple phytochemical extracts to inhibit cancer cell proliferation in vivo, and (3) to determine whether apple phytochemical extracts affect apoptosis in mammary cancer cells in vivo.

MATERIALS AND METHODS

Reagents. Acetone, wax, ethanol, and formalin (36%) were purchased from Fisher Scientific (Pittsburgh, PA). Hematoxylin, eosin, proteinase K, and DMBA were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in the study were of analytical grade.

Apple Extraction. The Red Delicious variety of apples was purchased from Cornell Orchards (Cornell University, Ithaca, NY). Fresh apples were cleaned and dried before extraction. Apples were extracted using the method reported previously in our laboratory (*16*, *21*). Briefly, 100 g of fresh weight of the edible part of apples was weighed and homogenized with chilled 80% acetone (1:2, w/v) using a chilled Waring blender for 5 min. The sample was then further homogenized using a Polytron homogenizer for an additional 3 min. The homogenates were filtered through Whatman no. 1 filter paper on a Büchner funnel under vacuum. The filtrate was evaporated at 45 °C until <10% of the initial volume remained. The apple phytochemical extracts were recovered with distilled water to a final concentration 2 g of fresh apples/mL. The apple phytochemical extracts were then freeze-dried and stored at −40 °C until use in the feeding study. Control extracts were prepared using the same extraction solvents and procedures without apples.

Animal Care and Treatment. Pathogen-free weanling female Sprague−Dawley rats were purchased at 30 days of age, adapted immediately to the AIN-76 diet, and housed in a room with a 12 h light/12 h dark cycle. The rats were acclimated to the surroundings in the animal room for 1 week prior to the initiation of the experiment. Care and treatment of rats followed the recommended guidelines of the National Research Council (1985). The rats were randomly assigned to five groups ($n = 30$/group). Four of five groups of rats (50 days old) were given 10 mg of DMBA dissolved in 1 mL of corn oil by gavage; the fifth group received no DMBA and was given 1 mL of corn oil, served as the negative control group. Rats were administered the control extracts or whole apple extracts starting 2 weeks prior to DMBA administration and continuing for 24 weeks until the end of the experiment. Three levels, low, middle, and high, of doses of whole apple extracts at 3.3, 10.0, and 20.0 g of fresh apples/kg of body weight (bw), respectively, which are equivalent to human consumption of 1, 3, and 6 apples per day, were given to the rats by gavage. Animals were weighed weekly and palpated twice a week to check for the development of palpable mammary tumors. The time of tumor appearance was also recorded.

Animals that died during the experiment were autopsied, and those becoming moribund were sacrificed for examination. All survivors were sacrificed under anesthesia at the end of the 24th week after DMBA administration. All animals were subjected to complete autopsy. At the time of necropsy, all tumors were removed and weighed, and tumor volume was recorded (*22*). The tumor incidence and cumulative tumor yield were calculated in each group. The tumor latency period in each group was expressed by the mean of tumor appearance time. All tumors were fixed in 10% neutral formalin solution and subsequently dehydrated and embedded in wax. The tissue wax was cut into 3−5 μm sections, fixed on slides, and processed for microscopy examination (stained with hematoxylin and eosin, HE) or immunohistochemistry analysis as described below. Histopathological evaluation was carried out on coded slides following the International Agency for Research on Cancer (IARC) classification of mammary rodent tumors (*23*).

Immunohistochemical Staining of PCNA, Cyclin D1, Bcl-2, and Bax. The sections of mammary cancer tissues were deparaffinized in xylene and rehydrated through graded alcohol. The sections were incubated for 10 min at 95−100 °C in 10 mmol/L sodium citrate buffer (pH 6.0). Endogenous peroxidases were inactivated by immersing the sections in 3% hydrogen peroxide for 10 min and then were incubated for 10 min with 10% normal goat serum to block nonspecific binding. The sections were subsequently incubated at 4 °C overnight with anti-PCNA antibody (monoclonal mouse, PC10, IgG, 1:50 dilution, Calbiochem Laboratory, Inc., Temecula, CA), or anticyclin D1 antibody (monoclonal mouse, DCS-6, IgG, 1:25 dilution, Calbiochem Laboratory, Inc.), or anti-Bcl-2 antibody (rabbit polyclonal, N-9, IgG, 1:15 dilution, Santa Cruz Biotechnology, Delaware, CA), or anti-Bax antibody (polyclonal rabbit, AB-1, IgG,1:20 dilution, Calbiochem Laboratory, Inc.). Then, the sections were incubated with biotinylated anti-mouse IgG or anti-rabbit IgG (ZYMED Laboratory, Inc., Carlsbad, CA) for 30 min, followed by peroxidase-conjugated streptavidin (ZYMED Laboratory, Inc.) for 30 min. The chromogenic reaction was developed with 3,3′-diaminobenzidine (DAB) for 3 min, and all sections were counterstained with hematoxylin. The same protocol was applied to the negative control with the omission of the primary antibody. Microscopic images were measured at a magnification of 400×. Two thousand cells were counted in five visual fields in each section, and an average of over 10 tissue masses randomly chosen from at least 5 rats in each group was analyzed to determine the cells positively stained for specific protein expression (mean ± SD).

Immunohistochemical Staining by TUNEL Assay. The effect of apple extracts on apoptosis in rat mammary cancer tissues was evaluated by TUNEL assay (Promega Corp., Madison, WI). The slices of mammary cancer tissues were deparaffinized in xylene and rehydrated through graded alcohol, then washed in 2% NaCl in phosphate-buffered saline (PBS) buffer, fixed in 4% paraformaldehyde, treated with proteinase K (20 μg/mL) for 30 min, refixed with 4% paraformaldehyde, and incubated with TdT reaction mix in a humidified box for 1 h at 37 °C. The slices were washed in 2× SSC (0.15 mol/L sodium chloride and 0.015 mol/L trisodium citrate, SSC solution) for 15 min and then blocked in 3% H_2O_2 for 5 min. The slides were then incubated with streptavidin−horseradish peroxidase (HRP) and colorized with DAB. All sections were counterstained with hematoxylin. Controls were subjected to the same protocol with the omission of the TdT reaction mix. Microscopic images were measured at a magnification of 400×. Two thousand cells were counted in five visual fields randomly in the section, and an average of over 10 tissue masses randomly chosen from at least 5 rats in each group was examined to determine the number of apoptotic cells, which were identified by a brown stain over the nuclei (mean ± SD).

Statistical Analysis. Data were expressed as mean ± SD. A one-way analysis of variance was used to analyze body weight. Fisher's exact test was used to compare the percentage of rats with tumors and tumor histology in each group. The expression of PCNA, TUNEL, cyclin D1, Bcl-2, and Bax in mammary gland tumors was analyzed using Student's *t* test, Welch's *t* test, or ANOVA. For the rats that were killed early because of tumor burden, we assumed that the number of tumors in killed rats remained constant until the end of the study. Data analyses were generated, and plots were constructed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL) and SigmaPlot version 10 for Windows (Systat Software Inc., San Jose, CA). Statistical significance was set at $P < 0.05$ and $P < 0.01$, and all P values were unadjusted for multiple comparisons.

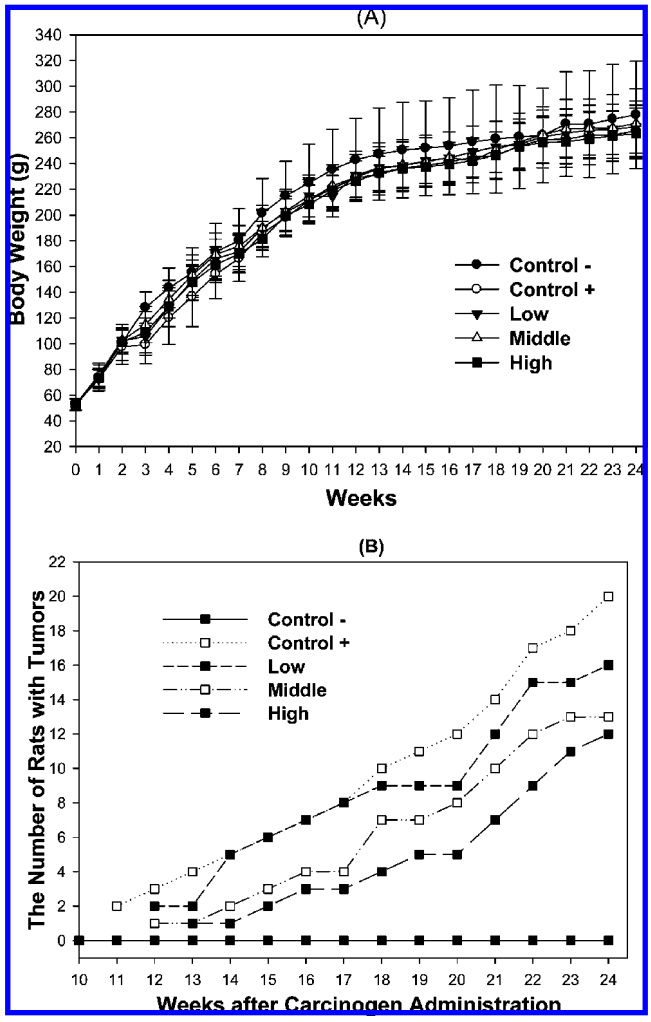

Figure 1. Mammary cancer prevention of whole apple extracts. Thirty-day-old female Sprague−Dawley rats were fed three levels of 3.3 (low), 10.0 (middle), and 20.0 (high) g of fresh apples/kg of body weight starting 2 weeks before DMBA administration and continuing for 24 weeks until the end of the experiment: (**A**) animal body weight of each group during the 24 weeks; (**B**) rats with observable mammary tumors. Control −, negative control group; control +, positive control group.

RESULTS

Effects of Apple Extracts on Body Weight and Main Organs of Rats. Body weights of animals in each group were monitored weekly. Throughout the experiment, there were no significant differences in body weight in animals fed apple extracts in comparison with the control groups ($P > 0.05$, **Figure 1A**). At the termination of the experiment at 24 weeks, the average final body weight and relative weight of organs (liver, spleen, kidneys, and ovaries) were not significantly different between the control groups and the groups fed apple extracts ($P > 0.05$) (data not shown). That suggests that there was no toxicity observed in the animals fed the apple extracts at the doses tested.

Effects of Apple Extracts on the Prevention of Mammary Carcinogenesis. In this study, SD rats were given the low, middle, and high doses of apple extracts starting 2 weeks before DMBA administration and continuing for 24 weeks. The positive control group, given a 10 mg dose of DMBA, developed tumors at 11 weeks. The treatment groups, given the low, middle, and high doses of apple extracts, developed tumors at 12, 12, and 13 weeks after carcinogen DMBA administration, respectively. The survival of rats without tumors in each group given the apple extracts was higher than that of the positive control group. Sequential observation data for incidences of palpable mammary tumors in the positive control and apple-treated groups and the total number of rats bearing tumors are presented in **Figure 1B** and **Table 1**. Final incidences of mammary tumors, including those from animals that died or were killed during the experiment, were significantly decreased by the apple treatments (tumor incidence: control, 71.4%; low, 59.3%; middle, 43.3%; high, 40.0%), and a clear dose dependence was observed. Application of low, middle, and high doses of whole apple extracts reduced tumor incidence by 17.0%, 39.4% ($P < 0.02$), and 44.0% ($P < 0.01$), respectively (**Table 1**). Cumulative tumor numbers in the control group and the three levels of low, middle, and high doses of apple extracts were 36, 27, 27, and 14, respectively, and were reduced by 25.0, 25.0, and 61.1% in comparison with the control group ($P<0.01$), after 24 weeks, respectively (**Table 1**). The tumor latency periods after DMBA administration were 18.8 weeks for rats in the control group and 17.8, 19.8, and 21.0 weeks for rats in the low-, middle-, and high-dose groups, respectively. The delay of tumor onset was dose-dependent ($P > 0.05$) (**Table 1**).

The combined weight of all mammary tumors of each group in the control group and the three levels of low, middle, and high doses of apple extracts were 134.0, 75.3, 57.8, and 26.5 g, respectively, and were reduced by 43.8, 56.9, and 80.2%, respectively, in comparison with the control group ($P < 0.01$) (**Table 1**). The average volume of tumor in the positive control and apple-treated groups at the end of the experiment showed a downward trend (**Table 1**). The average weights of tumors per rat in the groups fed middle and high doses of apple extracts were significantly different in comparison with the control group ($P < 0.05$ and $P < 0.01$) (**Table 1**). The average weight of tumors per rat was reduced by 10.2, 54.8, and 69.4% in rats fed three levels of low, middle, and high doses of apple extracts, respectively, compared to the control group. The average weight of each tumor in rats fed the high dose of apple extracts was significantly different from the control group ($P < 0.05$) (**Table 1**).

Mammary Tumor Histopathology. Mammary tumor histology data and morphology are presented in **Table 2** and **Figure 2**, respectively. At the termination of the study, 81.3% (13/16), 56.5% (13/23), 50.0% (11/22), and 23.1% (3/13) of adenocarcinoma in the rat's neoplasm in the control and apple-treated groups, respectively, had been examined by HE. There was significant difference between control and middle and high doses of apple-treated groups ($P < 0.05$) (**Table 2**). The other types of tumors in mammary tissue were not different between the control and apple-treated groups. There was a decreasing rate of adenocarcinoma of mammary tissues following a trend of the groups of low, middle, and high doses of apple extracts (**Table 2**).

Immunohistochemistry for PCNA and Cyclin D1 Expression. Proliferating cell nuclear antigen (PCNA) is a common index for proliferation of mammary cancer cells at early and late G1 stage. To determine if apple extracts affected mammary gland cell proliferation, we analyzed the expression of PCNA in mammary cancer tissues by immunohistochemistry. As shown in **Figure 3I**, the expression of PCNA in mammary cancer tissues of apple-treated groups (309 ± 104, 76 ± 34, and 25 ± 15 per 2000 cells in low-, middle-, and high-dose groups, respectively) significantly decreased in comparison with the control group (614 ± 240 per 2000 cells) ($P < 0.05$). Both the positive group and the low dose of apple extracts group were significantly different in the expression of PCNA when com-

Table 1. Mammary Cancer Prevention of Whole Apple Extracts[a]

group	*n*	DMBA	dose (g/kg of bw)	tumor incidence (%)	cumulative tumor yield	tumor latency period (WK)	total tumor wt (g/group)	tumor wt per rat (g)	av vol (cm^3/tumor)	av wt per tumor (g)
control −	30	−	−	0.0	0	0.0	0	0 ± 0	0 ± 0	0 ± 0
control +	30	+	−	71.4	36	18.8	134.0	4.79 ± 5.90	1.57 ± 2.02	3.72 ± 2.72
low	30	+	3.3	59.3	27	17.8	75.3	2.79 ± 5.00	1.41 ± 3.18	2.79 ± 3.64
middle	30	+	10.0	43.3*	27	19.8	57.8	1.93 ± 4.58*	0.71 ± 1.13	2.14 ± 2.34*
high	30	+	20.0	40.0*	14*	21.0	26.5	0.88 ± 1.80**	0.48 ± 0.66	1.89 ± 1.95*

[a] *, $P < 0.05$, and **, $P < 0.01$, compared to the corresponding positive control group. Control −, negative control group; control +, positive control group.

Table 2. Classes of Mammary Tumors in the Control and Apple-Treated Groups[a]

group	no. of tissues	adenocarcinoma (%)	adenoma (%)	fibroadenoma (%)	benign masses (%)
control +	16	13 (81.25)	1 (6.25)	2 (12.50)	0 (0.0)
low	23	13 (56.52)	6 (26.09)	0 (0.0)	4 (17.39)
middle	22	11 (50.00)*	6 (27.27)	2 (9.09)	3 (13.64)
high	13	3 (23.08)**	6 (46.16)*	2 (15.38)	2 (15.38)

[a] *, $P < 0.05$, and **, $P < 0.01$, compared to the control group. Control +, positive control group.

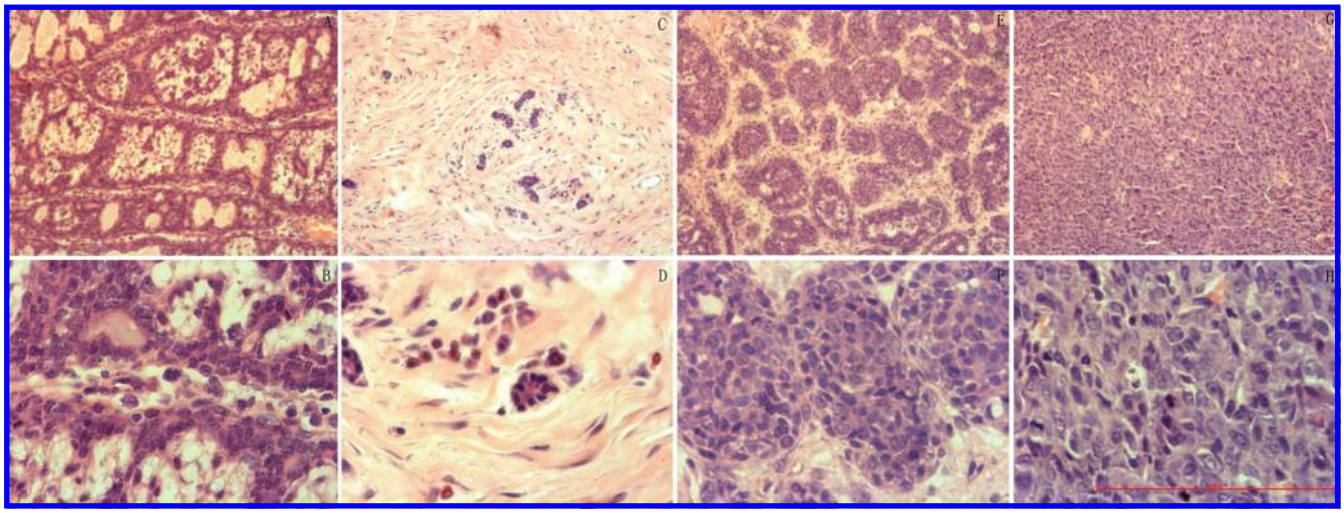

Figure 2. Histopathological findings in mammary tumors stained with HE: (**A**, **B**) HE-stained section of a mammary adenolipoma (10× and 40×); (**C**, **D**) HE-stained section of a fibroadenoma (10× and 40×); (**E**, **F**) HE-stained section of an adenoma (10× and 40×); (**G**, **H**) HE-stained section of a moderately differentiated adenocarcinoma (10× and 40×). The neoplastic cells show great diversity in size and arrangement around the lumens, becoming multilayered and palisaded and showing papillary or even disorganized or solid growth. Their nuclei are enlarged, euchromatic with multiple nucleoli. The cytoplasm is scant, and mitotic figures are common.

pared to the middle- and high-dose groups ($P < 0.01$). The inhibitory rates of PCNA expression were 50, 88, and 96% in the three levels of low, middle, and high doses of apple extracts, respectively, in comparison with the control group and showed a dose-dependent manner (**Figure 3I**).

Cyclin D1 protein expression was detected among the mammary cancer tissues in the control and apple-treated groups by immunohistochemistry. As shown in **Figure 3II**, the expression of cyclin D1 in mammary cancer tissues in the control group (43 ± 35 per 2000 cells) was significantly different from that in the apple-treated groups ($P < 0.05$). The inhibitory rates of cyclin D1 expression in the apple-treated groups were 30, 65 ($P < 0.05$), and 84% ($P < 0.05$) in the three levels of low, middle, and high doses of apple extracts, respectively, when compared to the control group. A dose response was also observed.

Expression of Apoptosis, Bcl-2, and Bax in Mammary Cancer Tissues. As shown in **Figure 4I**, occurrence of apoptosis in mammary cancer tissues in the high dose of apple extracts group was significantly higher than those in low, middle, and control groups ($P < 0.05$). The middle-dose group also showed significantly different apoptosis expression from the control and high-dose groups ($P < 0.05$). Apoptosis expression was increased by 30 times in the high-dose group when compared to the control group. Apoptosis was induced by the apple extracts in a dose-dependent manner.

The expression of Bcl-2 and Bax in mammary cancer tissues was also investigated by immunohistochemistry. The expression of Bcl-2 protein in the control group (71 ± 74) was significantly different from that in low, middle, and high doses of apple extracts (67 ± 35, 22 ± 15, and 8 ± 14 per 2000 cells, respectively) ($P < 0.01$) (**Figure 4II**). The inhibitory rates of Bcl-2 expression were 6, 69, and 89% in low, middle, and high doses of apple extracts, respectively, in comparison with the control group ($P < 0.01$). The expression of Bax in the high-dose group (275 ± A 225 per 2000 cells) was significantly different from the other groups including the control group ($P < 0.01$) (**Figure 4III**) and was 7 times greater than that in the control group. In this animal model, the apple treatments inhibited the expression of Bcl-2 and induced the expression of Bax in a dose-dependent manner (**Figure 4II**,**III**).

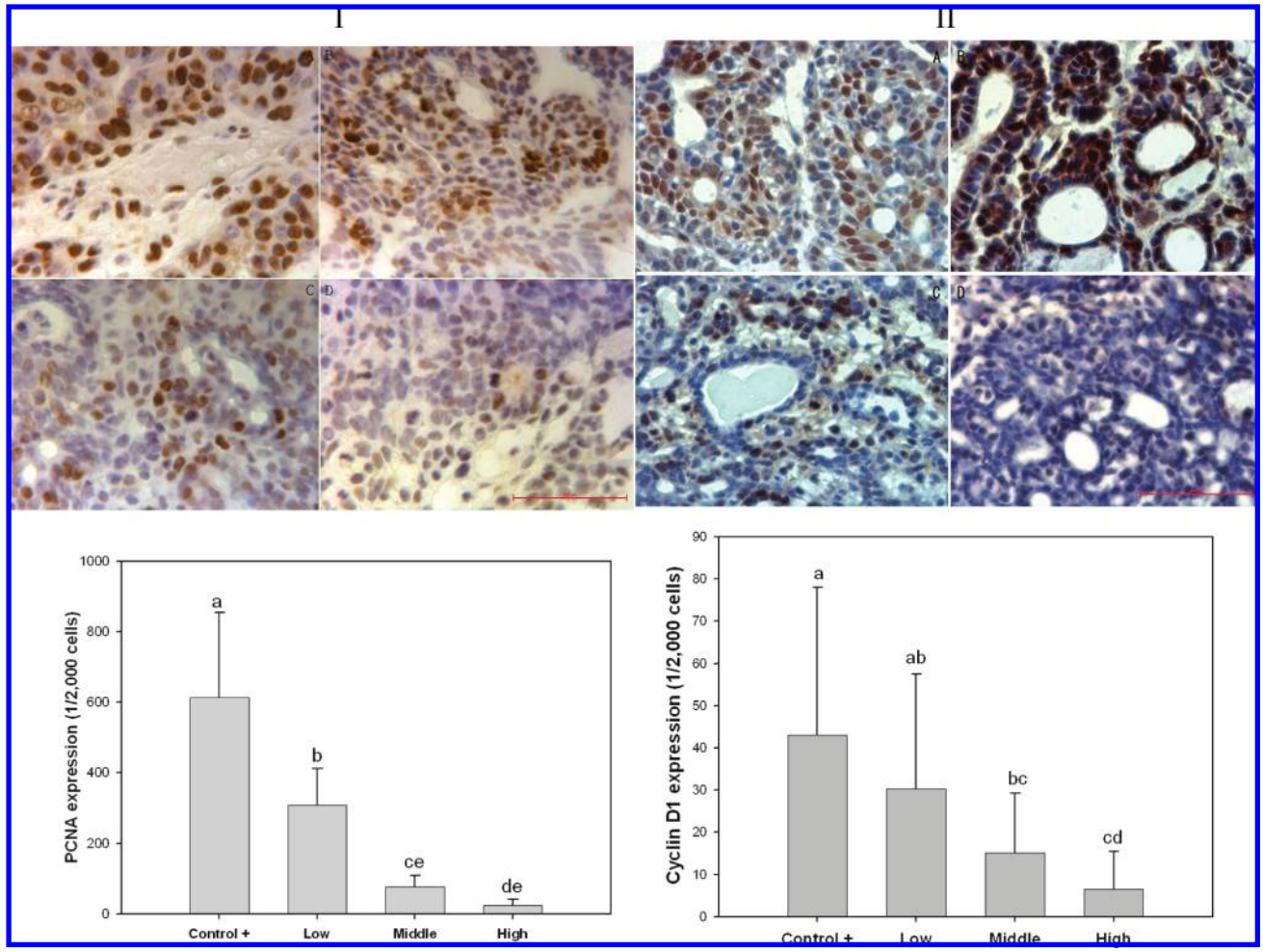

Figure 3. Effects of different doses of whole apple extracts on expression of PCNA (**I**) and cyclin D1 (**II**) in the mammary cancer tissues in vivo. High-power view (400×) of PCNA and cyclin D1 expression: (**A**) positive control group (control +); (**B**) low dose (low); (**C**) middle dose (middle); (**D**) high dose (high). The expressions of PCNA and cyclin D1 were visualized with DAB; positive cells are reddish-brown in color. Bars with no letters in common are significantly different (P<0.05).

DISCUSSION

Phytochemicals, the bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods, have been linked to reduction in the risk of major chronic disease including cancer (*8, 9*). More and more convincing evidence suggests that the health benefits of phytochemicals in fruits and vegetables may be even greater than is currently understood. Block et al. (*7*) have established this in an epidemiologic review of over 200 studies that examined the relationship between fruit and vegetable intake and cancers of the lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder, pancreas, and ovary. In 128 of 156 dietary studies, the consumption of fruits and vegetables was found to have a significant protective effect.

In the previous studies of selected common fruits and vegetables, apple has been shown to possess potent antioxidant activity and antiproliferation activity against cancer cells (*16, 19*). Median effective doses (EC_{50}) were 42.5 ± 2.6 and 49.4 ± 1.6 mg/mL in human colon cancer Caco-2 cells and liver cancer $HepG_2$ cells treated with different levels of whole apple extracts (*24*). Gosse et al. (*25*) reported that apple procyanidins had chemopreventive properties in a model of colon cancer and affected intracellular signaling pathways and triggered apoptosis in a human adenocarcinoma-derived metastatic cell line (SW620). Another study (*26*) reported that apple procyanidins in drinking water also prevented colon carcinogenesis in male Wistar rats induced by azoxymethane (AOM). The mucosal surface of the colon of rats receiving apple procyanidins showed a significant reduction in the number of preneoplastic lesions initiated by AOM ($P < 0.01$). The number of aberrant crypt foci (ACF) and the total number of aberrant crypts were reduced by 50% in rats given apple procyanidins.

In this study, female Sparague−Dawley rats were fed three levels of 3.3 (low), 10.0 (middle), and 20.0 (high) g of fresh apples/kg of body weight per day starting 2 weeks before DMBA administration and continuing for 24 weeks until the end of the experiment. The positive control group with carcinogen DMBA developed mammary tumors with 71.4% tumor incidence during a 24-week study, and no tumors were detected in the negative control group without DMBA. A dose-dependent inhibition of mammary carcinogenesis by whole apple extracts was observed (**Figure 1B**). These results demonstrate for the first time that whole apple extracts have potent anticarcinogenic activity of mammary cancer induced by DMBA in a rat model and that tumor incidence was decreased in a dose-dependent manner during the 24-week study. We have proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their potent antioxidant and anticancer activities and that the benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals present in whole foods (*8, 9*). This hypothesis partially explains why no single antioxidant can replace the

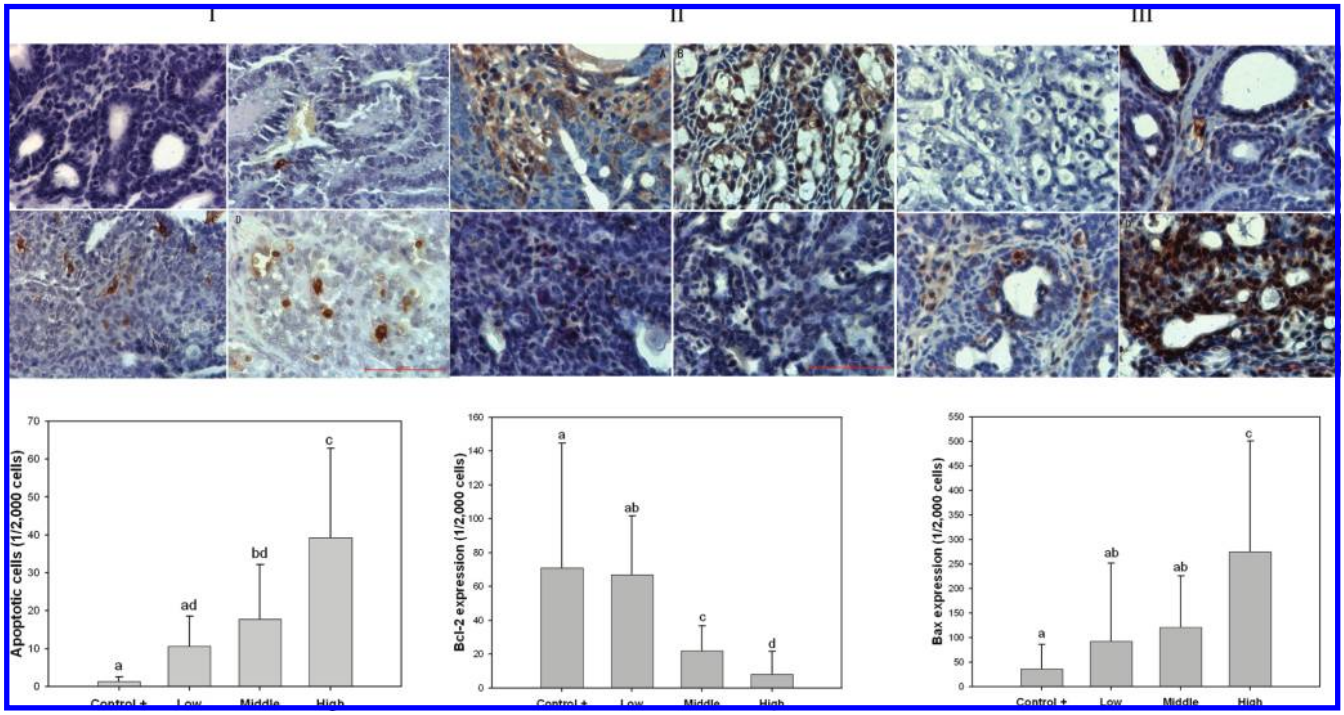

Figure 4. Effects of different doses of whole apple extracts on apoptosis (**I**) and expression of Bcl-2 (**II**) and Bax (**III**) in the mammary cancer tissues in vivo: (**A**) positive control group (control +); (**B**) low dose (low); (**C**) middle dose (middle); (**D**) high dose (high). Apoptotic cells were observed by TUNEL assay (**I**). The apoptotic cells in mammary cancer tissues were observed as the brown color stained in the nucleus. The expression of Bcl-2 and Bax was detected as the brown color stained in the cytoplasma. Bars with no letters in common are significantly different (P<0.05).

combination of natural phytochemicals in fruits and vegetables in achieving ultimate health benefits.

The main histopathological differential diagnoses in the mammary cancer tissues include benign (hyperplasia and adenolipoma), fibroadenomas, adenomas, and adenocarcinomas based on the IARC classification of rodent tumors (*23*). The type adenocarcinoma is a kind of highly malignant tumor, the main cause of death in mammary cancer animals and breast cancer patients, and is a main target for chemotherapy. Adenocarcinoma is always accompanied by the occurrence of distant metastasis and areas of necrosis, ulceration, and hemorrhage (*27*). Pathological findings in this study showed that there was 81.3% adenocarcinoma in the control group and 56.5, 50.0, and 23.1% in the three levels of low, middle, and high doses of apple extracts, respectively, during the 24-week study (P < 0.05).

We further determined whether the whole apple extracts affected cell proliferation and cell cycle in mammary cancer tissues of rats. The cell proliferation in mammary cancer tissues was measured with PCNA as a proliferative marker and cyclin D1 as a factor related to cell cycle. PCNA is a proliferating cell nuclear antigen, which functions as an auxiliary protein for DNA polymerase δ and is required for DNA synthesis, and is a key protein in regulating DNA replication and DNA damage repair. PCNA is a stable cell-cycle regulated nuclear protein that is expressed during the cell cycle; its rate of synthesis is correlated directly with the proliferative rate of cells (*28*). PCNA is an early marker of cell proliferation in cancer and expressed in cells that have entered the cell cycle and can be used to assess the proportion of the cells in a tumor that are proliferating. Cyclin-dependent kinases (Cdks) are key cell cycle regulators, and their activities are modulated by binding to cyclins (*29*). Binding of cyclin D to cdk4 and cdk6 leads to the phosphorylation of the retinoblastoma (Rb) protein. Phosphorylation of Rb prevents it from repressing the E2F family of transcription factors and leads to the transcription of several genes required for the G1-to-S phase transition, thereby promoting cellular proliferation (*30*). Cyclin D1 is overexpressed in 35−50% of breast cancers (*31*). Because of its pivotal role in promoting cell cycle progression and, hence, cell division and proliferation, such overexpression might be expected to coincide with poor prognosis (*31*). Recently, we reported apple extracts markedly inhibited cell proliferation in both human breast cancer MCF-7 and MDA-MB-231 cells in a dose-dependent manner. The antiproliferative activity of apple extracts against MCF-7 and MDA-MB-231 cells could result from the induced G1 arrest with decreased expression of cyclin D1, Cdk4, and ppRb proteins (*32*). In this study, we observed that whole apple extracts demonstrated potent antiproliferative activity and affected the cell cycle in mammary cancer tissues. The expressions of PCNA and cyclin D1 were significantly decreased in mammary cancer tissues among the apple-treated groups when compared to the positive control group (P < 0.01 and P < 0.05) (**Figures 3I**,**II**) in a clear dose-dependent manner. We could at least partly explain that the cell proliferation in mammary cancer tissues was inhibited by down-regulation of the expression of PCNA and cyclin D1 to affect the cell cycle of tumor cells. We would further determine how apple treatment affects other cell cycle components in mammary cancer.

We also determined whether apoptosis was induced by whole apple extracts and their possible mechanism in induction of apoptosis in mammary cancer tissues. The cell apoptosis in mammary cancer tissues was detected by TUNEL assay with immunohistochemistry. In contrast to nonspecific cellular necrosis, apoptosis is characterized be a specific pattern of DNA degradation that exposes 3′-OH ends. As a result, apoptotic cells within tissue sections can be identified by TUNEL of exposed 3′-OH ends with nonisotopically digoxigenin-labeled dUTP and distinguished cellular necrosis (*33*). In this study, our findings showed that cell apoptosis was induced in mammary cancer

tissues of rats fed three levels of apple extracts and an evident dose−response relationship was observed (**Figure 4I**). Bcl-2 family members are pivotal regulators of the apoptotic process. The expressions of Bcl-2 and Bax were investigated in mammary cancer tissues to explain how to affect the pathway of apoptosis in the control and whole apple extracts treated groups. Bcl-2 family proteins mainly involve the cell-intrinsic apoptotic pathway by activation of pro-apoptotic Bcl-2 family members, which induce the permeabilization of the outer mitochondrial membrane (OMM), resulting in the release of cytochrome *c* (Cyt-*c*) and other intermembrane space proteins (*34*). Bcl-2 protein is an antiapoptotic protein, which prevents cell apoptosis, and Bax protein, a pro-apoptotic protein, on the contrary, promotes cell death. In our study, the expression of Bcl-2 was significantly decreased in the apple-treated groups when compared to the control group ($P < 0.01$) (**Figure 4II**). The expression of Bax protein tended to increase in the apple-treated groups in comparison with the control group ($P < 0.05$) (**Figure 4III**). The dose response was also observed in the expression of both Bcl-2 and Bax in mammary cancer tissues. Therefore, apoptosis induced by whole apple extracts was partly due to the down-regulation of the Bcl-2 expression and up-regulation of the Bax expression in mammary cancer tissues.

In a previous study, apple extracts, in doses of >2000 mg/kg, were used to feed rats in a 90-day consecutive oral administration toxicity test, and the no observed adverse effect level (NOAEL) of apple phenolics compounds was found (*35*). In our study, rats were fed whole apple extracts with 3.33, 10.0, and 20.0 g of fresh apple/kg of body weight per day, and animal body weight and food intake were not affected throughout the 24-week duration when compared to the control groups. All of this indicated there was no toxicity of apple extracts at any of the doses tested in our study (**Figure 1A** and **Table 1**).

In summary, our data suggested that whole apple extracts possess potent activity to suppress DMBA-induced mammary carcinogenesis in a rat model and that this suppression is at least partly attributed to both the inhibition of cell proliferation and induction of apoptosis. The inhibition of cell proliferation and induction of apoptosis in mammary cancer may be regulated through the down-regulation of cyclin D1 and Bcl-2 expression as well as the up-regulation of Bax expression. Thus, these findings strongly support the view that consumption of whole apples is an effective way to achieve cancer prevention as well as support our previous hypothesis that health benefits of fruits and vegetables are due to the additive and/or synergistic effect (*9*). Research should be done to determine the specific compounds recently isolated from apple peels (*36*−*38*). Further research is needed to determine the exact mechanism(s) of how apples prevent mammary cancer (*18*, *32*, *38*).

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