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Cranberry Phytochemical Extract Inhibits SGC-7901 Cell Growth and Human Tumor Xenografts in Balb/c *nu*/*nu* Mice

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Cranberry extract possesses potent antioxidant capacity and antiproliferative activity against cancer in vitro and in vivo. The objectives of this study were to determine whether the cranberry extract inhibited proliferation of human gastric cancer SGC-7901 cells and human gastric tumor xenografts in the Balb/c *nu*/*nu* mouse. Cranberry extract at doses of 0, 5, 10, 20, and 40 mg/mL significantly inhibited proliferation of SGC-7901 cells, and this suppression was partly attributed to decreased PCNA expression and apoptosis induction. In a human tumor xenograft model, the time of human gastric tumor xenografts in the mouse was delayed in a dose-dependent manner. A dose−response inhibition was also observed in the averages of size, weight, and volume of tumor xenografts in the mouse between the control and cranberry-treated groups. These results demonstrate fresh cranberries to be a chemopreventive reagent.

KEYWORDS: Cranberry; SGC-7901 cell; cell proliferation; apoptosis; tumor xenografts; gastric cancer; diet; cancer; Balb/c *nu*/*nu* mice

INTRODUCTION

Cancer is a major public health problem throughout the world. It is estimated that approximately 21500 new cases will be diagnosed and more than 10880 people will die from gastric cancer in the United States alone in 2008 (*1*). The survival rate of gastric cancer is very poor, usually less than 15% at 5 years (*2*). Despite a global decline in incidence and mortality, gastric cancer is still the fourth most common cancer and the second leading cause of cancer deaths worldwide (*2*). Gastric cancer is also one of the most common cancers in China. Although diagnosis and treatment are the major strategies of controlling cancer, cancer chemoprevention is one of the best strategies to prevent cancer.

Although epidemiological studies have consistently shown that a diet rich in fruits and vegetables is associated with a lower risk of cancer (*3*−*7*), the effect of a high consumption of fruits and vegetables on the incidence of gastric cancer remains controversial in many studies (*8*−*10*). Only two of the five case-control studies (*11*, *12*) and two of four cohort studies (*13*, *14*) found a significant protective role of fruits and vegetables in the etiology of gastric cancer. On the basis of the finding that a high consumption of fruits and vegetables is inversely related to the risk of gastric cancer (*15*), scientists have suggested that possible anticarcinogenic mechanisms of phytochemicals in fruits and vegetables are primary contributors to the health benefits in the prevention of cancers (*16*). Cranberry extract or cranberry constituent health benefits have been ascribed to antiviral activity (*17*), antibacterial activity (*18*), antioxidant activity (*19*, *20*), cardioprotective properties (*21*), and anticancer activities (*19*, *22*−*24*). In previous studies, cranberry extract had potent antioxidant capacity and inhibited the growth of mammary, colon, and liver cancer cells in vitro in a dose-dependent manner (*19*, *22*) and induced apoptosis of MCF-7 breast cancer cells (*22*).

However, the exact mechanism of gastric cancer inhibition by cranberry is still unclear. The objectives of the present study were to determine whether cranberry phytochemical extract (1) inhibited cell proliferation in human gastric cancer SGC-7901 cells, (2) induced apoptosis in SGC-7901 cells, and (3) inhibited human gastric tumor xenografts in Balb/c *nu*/*nu* mice.

MATERIALS AND METHODS

Chemicals. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). A mixture of ethylenedinitriletetraacetic acid (EDTA) and trypsin was purchased from Sigma Chemical Co. (St. Louis, MO).

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Cranberry Extraction. Fresh cranberries of the Stevens variety were cleaned and dried before extraction. Cranberries were extracted using the method previously reported (*19, 25*). Briefly, 100 g of fresh weight of the edible part of cranberries 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 approximately 90% of the filtrate had been evaporated. The cranberry extract were frozen and stored at −40 °C until used in the experiments.

Cell Culture. Human gastric adenocarcinoma SGC-7901 cell line was obtained from the Cancer Research Institute of Heilongjiang (China). SGC-7901 cells were cultured in RPMI 1640 (Gibco, Life Technologies, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37 °C with a humidified atmosphere of 95% air and 5% CO_2. The cells were subcultured with a mixture of EDTA and trypsin. Cells were plated at a density of 1×10^6 cells per 100 mm culture dish and allowed to grow to approximately 70% confluence before experimentation. To obtain constant results, several frozen stock ampules of SGC-7901 cells were not passaged more than 10 times after cells had been recovered from liquid nitrogen.

Cell Growth and Viability Assay. The effect of cranberry on cell viability was determined by MTT assay. Briefly, SGC-7901 cells were plated at 2.5×10^4 cells per well in 96-well microtiter plates. After 24 h, cells were treated with 200 μL of complete culture medium containing 1, 5, 10, 20, 30, 40, and 50 mg/mL of cranberry extract or with a vehicle control (medium control). Each dose of cranberry was repeated in triplicate. After incubation for 48 and 72 h, cell viability was determined. Twenty microliters of MTT [5 mg/mL in phosphate-buffer saline (PBS) stock, diluted to a concentration of 0.5 mg/mL with media] was added to each well and incubated for 4 h. After careful removal of the medium, 100 μL of DMSO was added to each well and shaken carefully. The absorbance was recorded on a microplate reader (Dynex Technologies, Chantilly, VA) at a 570 nm wavelength. The effect of cranberry on cell growth inhibition was assessed as percent cell viability, where vehicle-treated cells were taken as 100% viable.

Proliferating Cell Nucleus Antigen (PCNA) Expression in SGC-7901 Cells. The expression of PCNA was examined by immunocytochemistry. Briefly, 3×10^5 cells were plated on a Falcon 8 chamber culture slide (Becton Dickinson Labware, Franklin Lakes, NJ). After reaching 60−70% confluence, the cells were serum-starved overnight without FBS medium. The cells were then exposed to 0, 5, 10, 20, and 40 mg/mL cranberry extract for 48 h. The cells were washed with PBS and fixed in cold acetone for 10 min at room temperature. Endogenous peroxidase was quenched for 10 min with 3% H_2O_2 at room temperature. The sections were then incubated for 10 min with 10% normal goat serum to block nonspecific binding. The sections were subsequently incubated overnight at 4 °C with anti-PCNA antibody (monoclonal mouse, Calbiochem Laboratory, Inc., Temecula, CA). Then, the sections were incubated with biotinylated anti-mouse IgG (ZhongShan, Inc., Beijing, China) for 30 min, followed by peroxidase-conjugated streptavidin (ZhongShan, Inc.) for 30 min. The chromogenic reaction was developed with 3,3′-diaminobenzidine (DAB) for 3 min, and all sections were counterstained with hematoxylin. In controls, the primary antibodies were omitted. Microscopic images were measured at a total magnification of 400×. One thousand cells were counted in five visual fields in each section and were analyzed to determine if the cells positively stained for specific protein expression (mean ± SD).

Determination of Cell Apoptosis. Cell apoptosis was determined by the ApopTag Plus Peroxides *In Situ* Apoptosis Detection Kit (ZhongShan, Inc.) based on the TUNEL assay. Briefly, 3×10^5 cells were plated on a Falcon 8 chamber culture slide. After reaching 60−70% confluence, the cells were serum-starved overnight without FBS medium. The cells were then exposed to 0, 5, 10, 20, and 40 mg/mL cranberry extracts for 48 h and then washed with PBS and fixed in cold acetone for 10 min at room temperature. Endogenous peroxidase was quenched for 15 min with 3% H_2O_2 at room temperature. Cell DNA fragments were labeled with peroxidase, which reacted with the peroxidase substrate, DAB, to give a permanent, localized brown stain. Cells were then counterstained with hematoxylin to further differentiate apoptotic cells from nonapoptotic cells. For each treatment, five fields, each containing 400 cells, were randomly selected for observation. Results were expressed as the percentage of apoptotic cells (mean ± SD) for three replications.

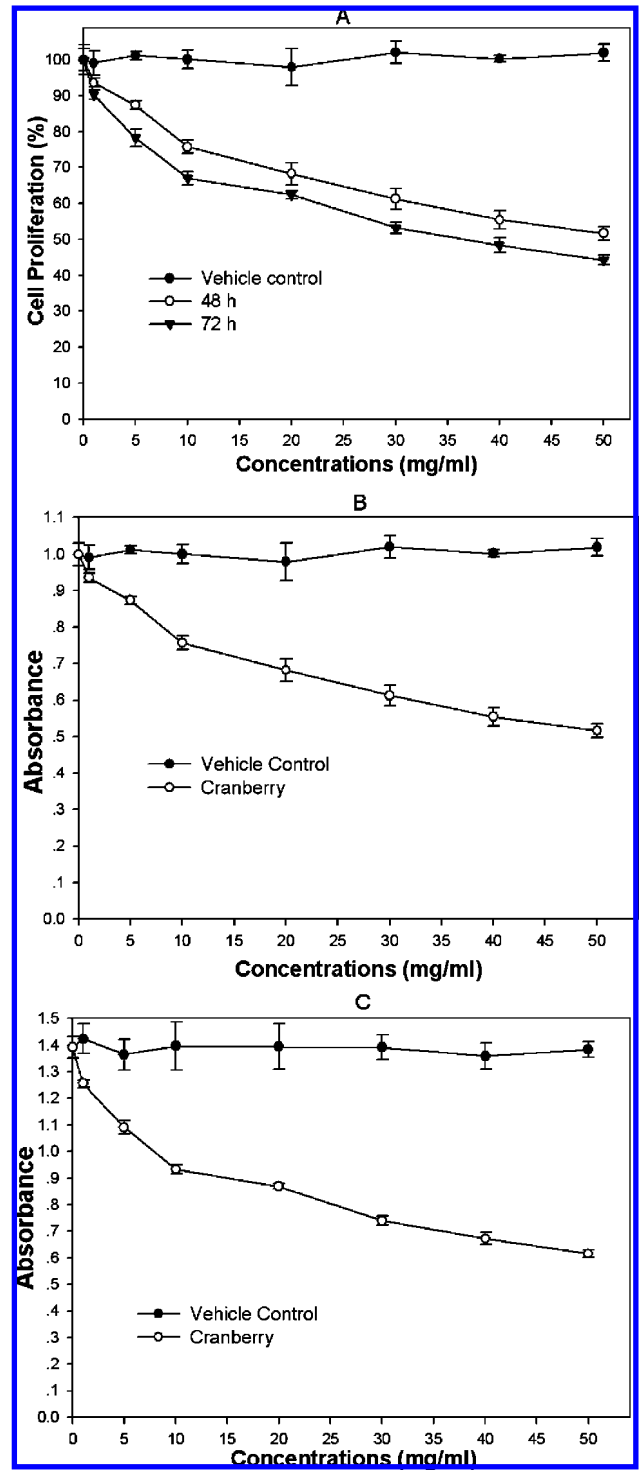

Figure 1. Effects of different doses of cranberry extract on cell proliferation of SGC-7901 cell: (**A**) cell growth curve; (**B**, **C**) actual absorbance values of cranberry and vehicle control groups for 48 and 72 h. SGC-7901 cells were plated at 2.5×10^4 cells per well in 96-well microtiter plates. After 24 h, cells were treated with 1, 5, 10, 20, 30, 40, and 50 mg/mL of cranberry extract or with a vehicle control for 48 and 72 h. Cell viability was determined by a microplate reader at a 570 nm wavelength.

Transmission Electron Microscopy. SGC-7901 cells from the negative control and cranberry treated (5, 10, 20, and 40 mg/mL for 48 h) groups were harvested, centrifuged, and washed with PBS at 4

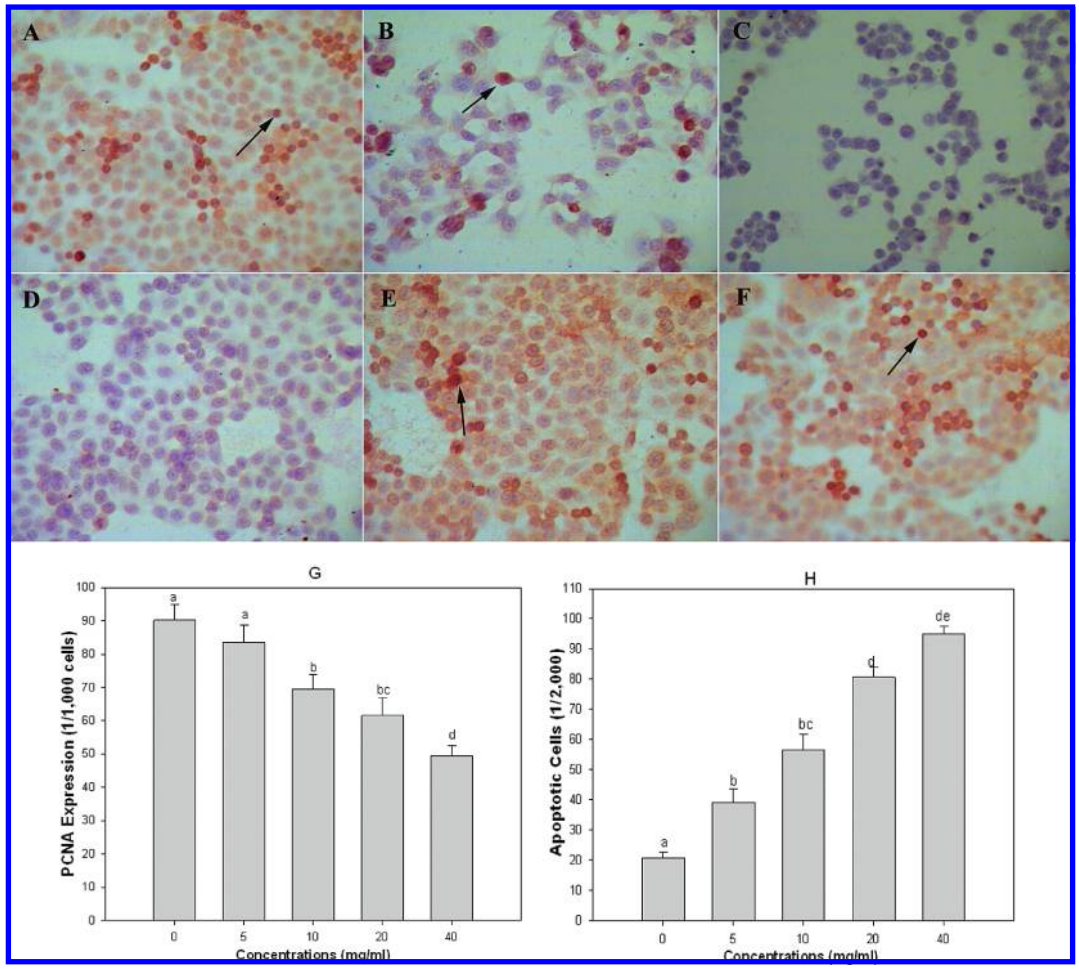

Figure 2. Effects of different doses of cranberry extract on PCNA expression and apoptosis induction. The expression of PCNA was visualized with DAB; positive cells are a reddish brown color in the nucleus. The apoptotic cells in SGC-7901 cells were observed as a brown color stained in the nucleus by TUNEL assay. (**A**−**C**) PCNA expression (×400), (**A**) expression of PCNA in the negative control group; (**B**) 10 mg/mL cranberry group; (**C**) 40 mg/mL cranberry group; (**D**−**F**) apoptosis determination by TUNEL assay (×400), (**D**) negative control group; (**E**) 10 mg/mL cranberry group; (**F**) 20 mg/mL cranberry group; (**G**, **H**) bars with no letter in common are significantly different ($P < 0.01$ or $P < 0.05$).

°C, and then the cell pellets were fixed with 2.5% glutaraldehyde in PBS for 2 h. After postfixation with 1% osmium tetroxide (OsO_4) in cacodylate buffer for 1 h at 4 °C, the pellets were dehydrated in graded ethanol solutions and embedded in Epon 812. Ultrathin sections of pellet were counterstained with uranyl acetate and lead citrate and observed under a transmission electron microscope (TEM).

Animal Care and Treatment. Four- to six-week-old BALB/c *nu*/*nu* mice were purchased from the Research Institute of Laboratory Animals at the Shanghai Chinese Academic Institute (Shanghai, China). The mice adapted immediately to the sterile basic supplementary diet and were housed in a room for 1 week with a 12 h light/12 h dark cycle prior to the initiation of the experiment. Care and treatment of mice followed the recommended guidelines of the National Research Council (1985). The mice were randomly assigned to five groups (n = 6/group), which were subcutaneously inoculated with 1.0 mL of SG-7901 cells (5×10^6 cells/mL in PBS) treated with 0, 5, 10, 20, and 40 mg/mL cranberry extract for 48 h in the right flank region. Animals were palpated daily to check for the development of palpable transplanting tumors. The time xenografts appeared was recorded. All animals were sacrificed under anesthesia at the end of the fourth week after inoculation of SGC-7901 cells. All tumors were removed and weighed. The length (diameter) (a) and width (b) of the tumor masses were measured, and the tumor volume (TV) was calculated as follows: $TV = (a \times b^2)/2$ (*26*).

Statistical Analysis. Data were expressed as mean ± SD. A one-way analysis of variance was used to analyze the diameter, weight, and volume of tumors. The expressions of PCNA and TUNEL in SGC-7901 cells were analyzed using Student's t test, Welch's t test, or ANOVA. Data analyses were generated, and plots were constructed using SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL) and SigmaPlot version 11.0 for Windows (Systat Software Inc., San Jose, CA). Statistical significance was set at $P < 0.05$.

RESULTS AND DISCUSSION

Effect of Cranberry Extract on SGC-7901 Cell Viability. Human gastric adenocarcinoma SGC-7901 cells were treated with medium containing cranberry extract (1−50 mg/mL) for 48 and 72 h. The cell viability of SGC-7901 cells treated with cranberry exract was evaluated by MTT assay. As shown in **Figure 1**, cell proliferation was significantly inhibited in SGC-7901 cells treated with different doses of cranberry extract for 48 and 72 h when compared to the vehicle group ($P < 0.05$ or $P < 0.01$). The EC_{50} values were 58.35 ± 6.38 and 37.12 ± 2.83 mg/mL for 48 and 72 h, respectively. A decrease of cell viability in a dose-dependent manner was observed (**Figure 1**). In previous studies of selected common fruits and vegetables, cranberry had been shown to possess a maximum potent antioxidant activity and the strongest capacity of antiproliferation in human hepatic cancer HepG2 cells and human mammary cancer MCF-7 cells treated with different levels of cranberry extract (*19*, *22*). Median effective doses (EC_{50}) were 14.5 ± 0.5 and 28.6 ± 6.1 mg/mL in HepG2 cells and MCF-7 cells

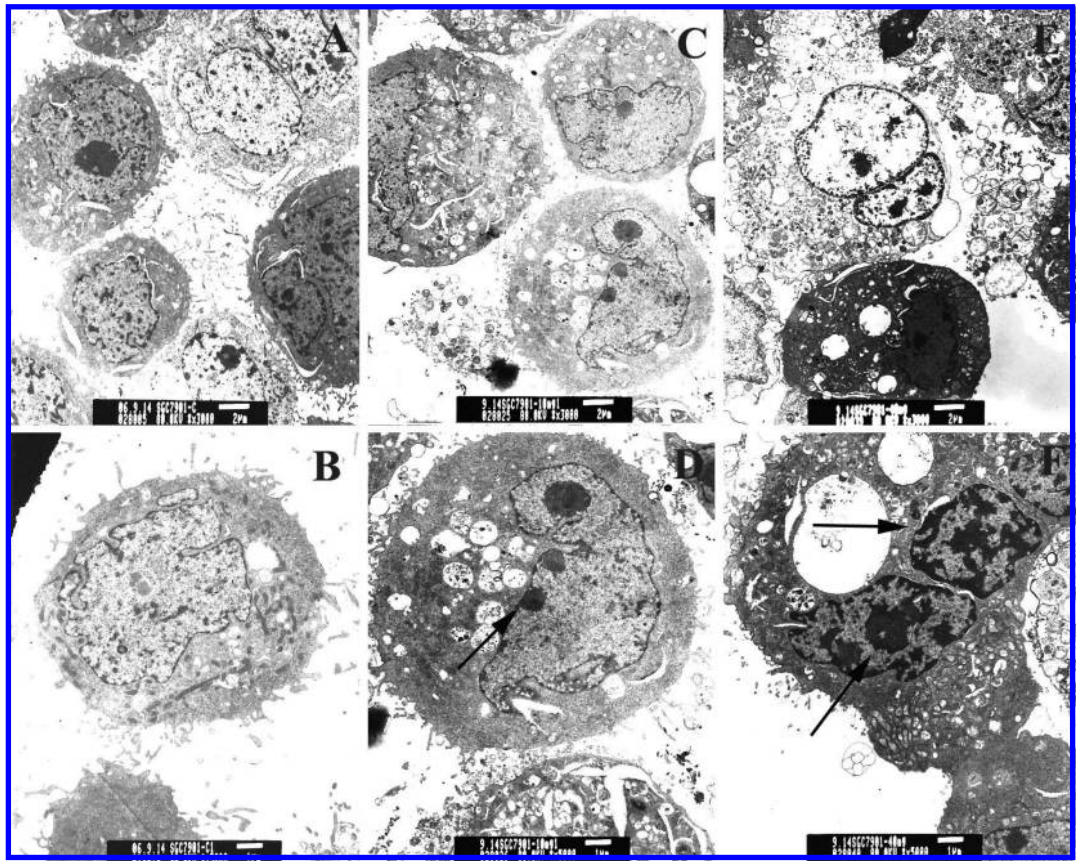

Figure 3. Observation of morphological changes by transmission electron microscope in SGC-7901 cells treated with different levels of cranberry extract. Characteritics of apoptosis are chromatin condensation, margination (arrowhead), shrinking cell body, cell blebbing, and vacuoles after SGC-7901 cells were treated by 10 (**C**, **D**) and 40 (**E**, **F**) mg/mL cranberry extract for 48 h compared to the control cells (**A**, **B**).

treated with different levels of cranberry extract. Thus, in our study, cranberry extract at doses of 1−50 mg/mL also significantly inhibited proliferation of SGC-7901 cell.

Expression of PCNA and Apoptosis Induction in SGC-7901 Cells. In this study, cranberry extract significantly inhibited cell proliferation in SGC-7901 cells. We are further determining whether cranberry extract could also inhibit the expression of PCNA in SGC-7901 cells. As shown in **Figure 2**, the expression of PCNA in SGC-7901 cells treated with different doses of cranberry extract was significantly lower than that in the negative control group in a dose-dependent manner ($P < 0.01$) (**Figure 2G**). The inhibitory rates were 7.3, 22.8, 31.7, and 45.1% in the 5, 10, 20, and 40 mg/mL dose groups, respectively, compared to the negative control group. PCNA is a nuclear protein that functions as an auxiliary protein for DNA polymerase δ and is an absolute requirement for DNA synthesis. PCNA, a key protein in DNA replication and DNA damage repair, 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 (*27*). As an early marker of cell proliferation in cancer, PCNA, if expressed in cells that have just entered the cell cycle, can be used to assess the proportion of the cells in a tumor that are proliferating. In the present study, cranberry extract also inhibited expression of PCNA in SGC-7901 cells.

Apoptosis, a noninflammatory process, plays an important role in numerous physiological and pathological events. Measurement of DNA fragmentation using the TUNEL assay is among the most widely used methods of detecting apoptosis (*28*). After exposure to cranberry extract, apoptotic induction was observed in SGC-7901 cells (**Figure 2**). In the control group, the apoptotic cells accounted for only 1.03% of the total cells. This value increased with treatments of cranberry extract in a dose-dependent manner (**Figure 2H**). The percentage of apoptotic cells in SGC-7901 cells treated with cranberry extract was significantly higher than that in the negative control group ($P < 0.05$ or $P < 0.01$). The rates of apoptotic cells were 1.9, 2.7, 3.9, and 4.6 times in the 5, 10, 20, and 40 mg/mL dose groups, respectively, in comparison with the negative control group.

Morphological changes were further observed by TEM in SGC-7901 cells treated with 5, 10, 20, and 40 mg/mL cranberry extract for 48 h (**Figure 3**). Characteristic morphological changes, including chromatin condensation, nuclear fragmentation, and shrinking cell bodies, were observed (**Figure 3E**,**F**). The cells showed obvious characteristic changes of apoptosis including cytoskeletal disruption, cell shrinkage, chromatin condensation, margination of nucelus, cell blebbing, formation of apoptotic bodies, and mitochondrial denaturation such as swelling and disappearance of mitochondrial cristae in the cranberry-treated cells (**Figure 3C**−**F**). In addition, cells in the control group had clear cell organs in the cytoplasm and mitochondrial cristae were also observed clearly (**Figure 3A**,**B**). In the previous study, cranberry phytochemical extract at doses from 10 to 50 mg/mL significantly inhibited cell proliferation of MCF-7 and HepG2 cells and arrested MCF-7 cells in the G0/G1 phase (*19*, *22*). Thus, in our study, cranberry extract inhibited cell proliferation of SGC-7901 cells, and this suppression may be attributed to decreased PCNA expression and increased nuclear fragmentation.

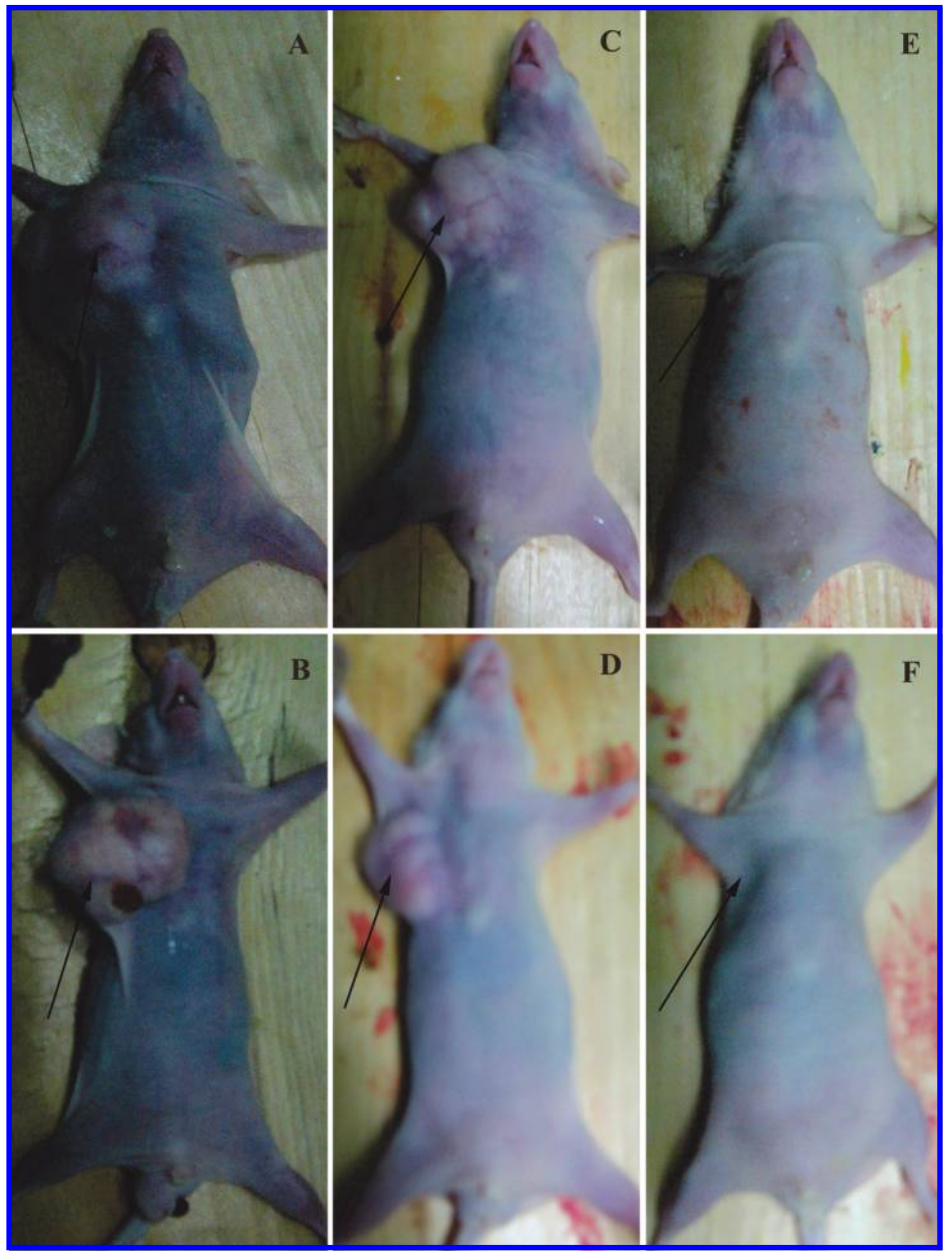

Figure 4. Effects of different doses of cranberry extract on human tumor xenografts in the BALB/c *nu*/*nu* mice: (**A**, **B**) control group; (**C**, **D**) 20 mg/mL group; (**E**, **F**) 40 mg/mL group. The arrowhead shows the appearance of human xenografts and the location of inoculation in the nude mice.

Table 1. Effects of Different Doses of Cranberry Extract on Human Tumor Xenografts in the BALB/c *nu*/*nu* Mice (Mean ± SD)[a]

cranberry extract (mg/mL)	*n*	av diameter (cm)	av tumor wt (g)	av tumor vol (cm^3)
40	6	0 ± 0	0 ± 0	0 ± 0
20	6	2.28 ± 0.14**	3.28 ± 0.12**	2.99 ± 0.15*
10	6	3.02 ± 0.10*	4.64 ± 0.17*	3.28 ± 0.128
5	6	3.86 ± 0.13	5.51 ± 0.11	4.71 ± 0.21
control	6	4.32 ± 0.17	6.42 ± 0.10	5.34 ± 0.13

[a] *, $P < 0.05$; **, $P < 0.01$, compared to the corresponding control group.

Effect of Cranberry Extract on a Xenograft Mouse Model. To determine whether cranberry extract inhibits mice bearing human tumor xenografts, we subcutaneously inoculated human gastric adenocarcinoma SGC-7901 cells into the flank region of BALB/c *nu*/*nu* mice. The control group developed human gastric tumor xenografts in mice on the 10th day. The treatment groups, 5, 10, and 20 mg/mL doses of cranberry extract, developed human tumor xenografts on the 13th, 17th, and 17th day after inoculation administration, respectively. However, the mice had not developed human tumor xenografts in the 40 mg/mL cranberry group after 28 days (**Figure 4**). Sequential observation data for averages of diameter, weight, and volume of human tumor xenografts in the control and treated groups are presented in **Table 1**. The averages of diameter, weight, and volume of human tumor xenografts in the cranberry-treated groups were significantly lower than those in the control group ($P < 0.05$ or $P < 0.01$). A dose−response relationship was observed. The diameter, weight, and volume of human tumor xenografts in the 20 mg/mL group were reduced by 47.2, 48.9, and 44.0%, respectively, compared to the control group.

Boateng et al. (*23*) reported chemopreventive properties for cranberry juice in a model of azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Fisher 344 male rats. The results showed a 77.2% inhibitory rate of ACF compared to the control group and an increased level of total glutathione-*S*-transferase (GST) activity in the liver of rats. Athymic nude mice (nu/nu)

are important models for understanding the maturation of T cells and their role in immunological responses. Although nude mice clearly show a marked deficiency in responses attributed to T cells, the development of T cell mediated antitumor immunity implies that extrathymic T cells are being stimulated and expanded (*29*, *30*). Extrathymic T cells may be important in antitumor protection, especially against epithelial cancer cells in subcutaneous or intradermal sites (*31*, *32*). Thus, the nude mouse may be a useful model for further dissecting those interactions crucial to establishing the T cell repertoire in euthymic individuals as well as elucidating the contribution of extrathymically derived T cells to the peripheral immune system (*33*). Concurrent with the higher number of T cells in general, an age-related decrease in transplantability of human tumor xenografts into BALB/c nude mice has been found (*34*). Thus, nude mice have been used to show regression of human tumor xenografts in preclinical studies of antitumor antibodies (*35*−*37*). Human tumor xenografts transplanted in athymic mice play a pivotal role in cancer research for the selection of candidates for chemopreventive agents (*35*). The particular molecular abnormality of cancer being targeted is shown to be present in a particular human tumor xenograft model (and, ideally, shown to be important in the proliferation of that tumor), and then implanted xenografts remain of significant value in the cancer drug discovery process (*36*). Furthermore, often in parallel with efficacy determinations, the xenograft model is useful in assessing the agent's pharmacokinetics and pharmacodynamics in that it provides a renewable and readily accessible source of target human tumor cells (*30*, *35*, *37*). In this study, SGC-7901 cells treated with different concentrations of cranberry extract for 48 h inhibited cell proliferation, decreased the expression of PCNA, and induced apoptosis. It suggested that there are big changes within cells including activation/inactivation of genes, changes of signal transduction, increasing/decreasing protein expressions of cell cycle and apoptotic pathways, etc. When the nude mouse was inoculated with SGC-7901 cells, human gastric tumor xenografts were developed. However, when SGC-7901 cells were treated with different concentrations of cranberry extract for 48 h and then the mice were inoculated with these cells, to some degree, human gastric tumor xenografts were decreased in the mouse. The time of formation of xenografts was delayed. A dose−response inhibition was observed in the averages of size, weight, and volume between the control and experimental groups. Surprisingly, we did not observe the growth of human gastric tumor xenografts inoculated with 40 mg/mL of cranberry extract. This suggests a big change in the characteristics of SGC-7901 cells treated with 40 mg/mL cranberry extract. Thus, cranberry extract significantly inhibited the growth of human gastric tumor xenografts in the nude mice. A mechanism of inhibition needs to be further studied.

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