Blueberries inhibit cyclooxygenase-1 and cyclooxygenase-2 activity in human epithelial ovarian cancer

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Abstract. Ovarian cancer (OC) is the sixth and eighth leading cause of cancer mortality among women in developed and developing countries, respectively. Medical therapy is the main method for the treatment of OC. However, drug toxicity and the marked side effects of chemotherapy limit the usage and therapeutic results of the treatments. Therefore, the identification of multi-target agents with few side effects and high effectiveness is required. Traditional Chinese medicine has been used clinically to treat various types of cancer for thousands of years and is considered to possess multiple components and agents, which exert efficient therapeutic functions with few side effects. Although blueberries have previously been used to treat various types of cancer, the effect on OC and precise molecular mechanism of function of the fruit remains unknown. Cyclooxygenase (COX)-1 and COX-2 have been reported to be the biomarkers of OC. Blueberries may affect the progression of OC by affecting COX levels. To investigate the issue, COX-1 and COX-2 were overexpressed or silenced in ovarian cancer SKOV3 cells. The effect of blueberries on SKOV3 cell viability was determined by an MTT assay. Furthermore, a mouse model for OC was established. The results indicated that blueberries inhibited the proliferation of OC cells by downregulating the levels of COX-1 and COX-2. Blueberry (400 mg daily) consumption reduced tumor size significantly in mice with OC compared with the control without blueberry treatment (P<0.05). The results suggest that blueberries should be used to develop a potential non-pharmaceutical therapy for OC.

Introduction

Ovarian cancer (OC) begins in the ovary and can result in abnormal cells invading numerous other parts of the body.

Symptoms are often vague and unapparent when the disorder begins. There are numerous symptoms for OC, such as bloating (1), pelvic pain (2) and abdominal swelling (3). A number of areas of the body can be affected by OC by the metastasis of abnormal cells, including the abdomen (4), bowel (5), bladder (6), lymph nodes (7) and liver (8). In 2012, there were 239,000 females affected by OC, which caused 152,000 mortalities worldwide (9). OC is the sixth and eighth leading cause of cancer mortality among women in developed and developing countries, respectively (10).

Medical therapy is the most widely used type of treatment for OC; examples include olaparib a biological therapy agent and taxol a chemotherapy agent (10-13). However, the majority of medicines used exhibit unwanted cell toxicity (14-17). Chemotherapy can cause different side effects, such as nausea and vomiting, distress, sexual dysfunction, fatigue and memory loss (18). The toxicity and side effects limit the usage and effectiveness of the aforementioned treatments for OC. It is therefore necessary to find an effective therapeutic method with fewer side effects for OC. Non-pharmaceutical treatment has become a novel therapy for various types of cancer. Blueberries (*Vaccinium spp*.), a type of fruit, have been identified to exhibit therapeutic effects on several types of cancer (19,20). For example, blueberries inhibit the progression of triple negative breast cancer and triple negative breast cancer-associated metastasis by the inhibition of inflammation via specific cytokine-mediated signaling pathways (21). Blueberries have been revealed to suppress tumor growth and metastasis (21), and are becoming an important fruit due to the associated chemopreventative and therapeutic potential against the tumorigenesis of numerous types of cancer. A blueberry-supplemented diet protects against 17β-estradiol-mediated mammary tumorigenesis (22). However, the molecular mechanisms of blueberries with respect to the inhibition of various types of cancer are unclear, and the therapeutic effect on OC remains unknown.

Cyclooxygenase (COX), termed prostaglandin-endoperoxide synthase (23), is an enzyme responsible for the formation of prostaglandins. A previous study reported the presence of high levels of COX-1 mRNA in high-grade serous ovarian cancer (24). COX-2 is also overexpressed in human ovarian cancer cells, and targeting COX-2 may be a potential approach for the treatment of OC (25). Blueberries may exhibit a therapeutic effect on OC, and COX-1 and COX-2 may be involved in the process. The present study aimed to explore

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the protective effect of a blueberry diet against the development of OC, which is hypothesized to act via the regulation of COX-mediated pathways.

Materials and methods

Materials. The blueberries were purchased from the Blueberry Production Field (Guizhou, China). The blueberries were frozen upon arrival at -20°C. A total of 100 g blueberries were homogenized in a homogenizer (GYB60-65; Shanghai Donghua High Pressure Homogenizer Factory, Shanghai, China). The blueberry juice was obtained by centrifuge at 10,000 x g, 40°C for 10 min (1 ml blueberry juice was produced from 2 g blueberry).

Cell culture. The human ovarian cancer SKOV3 cell line was purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured with McCoy's 5A medium without serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The medium was supplemented with 4 μg/ml transferrin (Chemicon International Inc.; EMD Millipore, Billerica, MA, USA), 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO, USA). and 10 ng/ml vascular endothelial growth factor (Chemicon International Inc.; EMD Millipore). The cells were cultured in 5% CO_2 at 37°C and 100% humidity, and transferred to fresh medium every 2 days.

Evaluation of cell viability by MTT assay. Cell viability was determined using an MTT assay. The SKOV3 cells were seeded into 96-well plates at a concentration of 1x10^4 cells per well in 100 μl McCoy's 5A medium. The cells were treated for 24, 48 or 72 h with 0, 1, 2, 4, 8 or 16 mg/ml blueberry juice. At the end of the culture, 5 μl MTT (10 mg/ml in PBS) was added to each well and incubated for an additional 3 h at room temperature. Untreated cells were used as control groups. The purple formazan was dissolved in dimethyl sulfoxide. Absorbance was recorded at 570 nm using an ELISA reader (Elisa Reader KD600; Shanghai Tongge Medical Devices Co., Ltd., Shanghai, China).

COX-1 and COX-2 expression construction. COX-1 and COX-2 genes were amplified and cloned into the NheI-EcoRI sites of pcDNA3.1 plasmid (Invitrogen; Thermo Fisher Scientific, Inc.), which were termed pcDNA3.1-COX-1 and pcDNA3.1-COX-2. The details of the CPX primers are as follows: COX-1 accession number, BC029840.1, sense, 5'-GTGAGCTAGCATGAGCCGGAGTCTCTTGC-3' and antisense primers, 5'-CTGAGAATTCTCAGAGCTCTGTGG ATGGTC-3', generating a 1820-base pair (bp) product; COX-2 accession number, AY462100.1, sense, 5'-GTGAGCTAGCAT GCTCGCCCGCGCCCTGC-3' and antisense primers, 5'-CTG AGAATTCCTACAGTTCAGTCGAACGTTC-3', generating an 1835-bp product. The constructed plasmids were amplified in *Escherichia coli* (Takara Biotechnology Co., Ltd., Dalian, China), isolated using a plasmid Miniprep Kit (Beijing TransGen Biotech Co., Ltd., Beijing, China) and were verified by sequencing (Takara Biotechnology Co., Ltd.). The classical Sanger sequencing was used. PCR reaction mixture was prepared using the following: 1 μl 100 ng plasmid, 1 μl primer (5 pmol; 5' Sequencing 1 Primer, CMV-F; 3' Sequencing

1 Primer, BGH-rev), 2 μl BigDye, 4 μl 5X buffer, 0.5 μl Taq DNA polymerase and ddH$_2$O added to 20 μl. The PCR reaction begins at 95°C 1 min, then 35 cycles of 96°C 10 sec, 52°C 15 sec and 72°C 1 min.

COX-1 and COX-2 RNAi. The lentivector pLKO.1 1 was purchased from Academia Sinica (Taipei, Taiwan). The siRNA sequence targeting COX-1, 5'-GTGAGCTATTACACT CGTATT-3' and COX-2, 5'-GATTGACAGTCCACCAAC TTA-3' was synthesized by Takara Biotechnology Co., Ltd., and linked to the lentivector pLKO.1, and therefore termed pLKO.1-COX-1-siRNA and pLKO.1-COX-2-siRNA. The SKOV3 cells were cotransfected by the reconstructed vectors by Lipofectamine® 2000 (Shanghai Yijie Biotechnology Co., Ltd., Shanghai, China) and pPACK Packaging Plasmid Mix (System Biosciences, Palo Alto, CA, USA).

Orthotopic implantation. All procedures were approved by the Animal Care and Ethics Committee of Guangdong General Hospital (Guangzhou, China). A total of 40 BALB/c 3-week-old nude mice (male:female=1:1; 15-20 g; height, 2-2.5 cm) purchased from the Animal Center of Guangdong Academy of Medical Sciences (Guangzhou, China) were raised in a high-efficiency particulate arrestance-filtered environment with a 12-h light/dark cycle. The SKOV3 cells were subcutaneously injected into 32 nude mice. At ~1 cm^3, the xenograft was excised and minced, and implanted into other 4-week old BALB/c nude mice according to the protocol of a previous study (26). A total of 4 days subsequent to the implantation of the OC tumors, the effects of blueberries on the mice was tested.

Experimental design. All the mice were assigned into either control or model group, with the model group being administered different concentrations of blueberry juice (100, 200 and 400 mg daily). The control and model groups were fed a normal diet and water, and all the model groups were administered a normal diet plus the corresponding blueberry juice. Subsequent to 2 weeks, the mice were sacrificed via cervical dislocation. Tumors were isolated under sterile conditions on a nutrient culture medium. Tumor size was determined using the ellipsoid formula v=4/3π abc, where v is volume, a and c are equal to half tumor height, and b is half tumor length. All the procedures for animal studies were consistent with the Animal Care and Use Guidance of Guangdong Academy of Medical Sciences (Guangzhou, China).

Quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR). Total RNA was purified from ovarian tumors of animal models or healthy mice using an RNA purification kit (cat. no. 74106; Qiagen, Inc., Valencia, CA, USA). Tumor tissues were cut into slices <2 mm and homogenized in 500 μl TRIzol. Phase was separated by the addition of 500 μl chloroform, and centrifuged at 12,000 x g for 10 min. The RNA was precipitated by adding 500 μl isopropyl alcohol. RNA was purified using a purification column. A total of 1 μg RNA from each sample was reverse-transcribed using the High-Capacity cDNA Reverse Transcription kit (cat. no. 4368813; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). A total of 1 μl purified RNA, poly(A)+-selected RNA primed with oligo
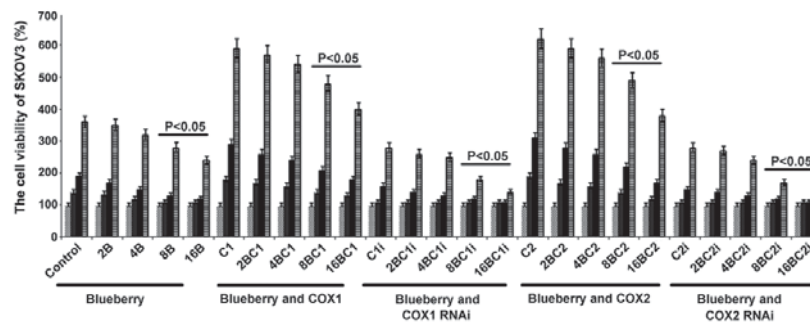
Figure 1. Blueberries inhibit cell growth rate of SKOV3 cells. SKOV3 cells were transfected with COX-1, COX-2, COX-1 RNAi and COX-2 RNAi, and treated with 0, 2, 4, 8 and 16 mg/ml blueberry juice. Cell concentrations were determined using MTT assays at days 0, 1, 2 and 3. The data represent the results of 5 independent experiments and are presented as the mean ± standard deviation. P<0.05 compared with 0 µg/ml blueberry subsequent to a 1-, 2- or 3-day culture. COX, cyclooxygenase; RNAi, RNA interference; Control, without transfection or blueberry; 2B, 2 mg blueberry; 4B, 4 mg blueberry; 8B, 8 mg blueberry; 16B, 16 mg blueberry; C1, COX-1; 2BC1, 2 mg blueberry and COX1 transfection; 4BC1, 4 mg blueberry and COX1 transfection; 8BC1, 8 mg blueberry and COX1 transfection; 16BC1, 16 mg blueberry and COX1 transfection; C1i, COX-1 RNAi; 2BC1i, 2 mg blueberry and COX1 RNAi; 4BC1i, 4 mg blueberry and COX1 RNAi; 8BC1i, 8 mg blueberry and COX1 RNAi; 16BC1i, 16 mg blueberry and COX1 RNAi; C2, COX-2; 2BC2, 2 mg blueberry and COX2 transfection; 4BC2, 4 mg blueberry and COX2 transfection; 8BC2, 8 mg blueberry and COX2 transfection; 16BC2, 16 mg blueberry and COX2 transfection; C2i, COX-2 RNAi; 2BC2i, 2 mg blueberry and COX2 RNAi; 4BC2i, 4 mg blueberry and COX2 RNAi; 8BC2i, 8 mg blueberry and COX2 RNAi; 16BC2i, 16 mg blueberry and COX2 RNAi.

(deoxy-thymidine) 100 nM, 1X annealing buffer and water were heated to 65°C for 5 min and placed on ice for 1 min. This reaction mix and 0.5 µl reverse transcriptase were added to the reaction for a final volume of 40 µl, and the mixture was incubated at 50°C for 50 min. The mixture was then heated to 95°C for 5. The SYBR Green DNA PCR kit was purchased from Applied Biosystems (Thermo Fisher Scientific, Inc.) and used for RT-qPCR using Applied Biosystems® 7500 Real-Time PCR Systems (Thermo Fisher Scientific, Inc.). The PCR primers were as follows: COX-1 forward, 5'-CAGAGCCAGATGGCT GTGGG-3' and reverse, 5'-AAGCTGCTCATCGCCCCAG G-3'; COX-2 forward, 5'-AAGTGCGATTGTACCCGGAC-3' and reverse, 5'-ACGTTCCAAAATCCCTTGAA-3'; GAPDH forward, 5'-AACTACATGGTTTACATGTT-3' and reverse, 5'-CACTTGATTTTGGAGGGATC-3'. After 1 min enzyme activation at 95°C, the reaction was cycled 45 times at 95°C for 10 s, 55°C for 10 s, and 72°C for 20 s. The values of the target genes were calculated utilizing the $2^{-\Delta\Delta Cq}$ method (27) for the fold change compared with GAPDH. The mRNA levels of the target genes were presented as relative increases compared with GAPDH. All experiments were performed in triplicate.

ELISA analysis. The concentrations of COX-1 and COX-2 in the treated and untreated samples were measured using Human COX-1 ELISA kit (cat. no. MBS026381) and Human COX-2 ELISA kit (cat. no. MBS043833; MyBioSource, San Diego, CA, USA), respectively.

Statistical analysis. Data were analyzed using one-way analysis of variance in SPSS 20 (IBM SPSS, Armonk, NY, USA). Quantitative data are shown as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of blueberry on the growth of SKOV3 cells. To investigate the effects of blueberries on SKOV3 cells, the concentration-dependent effects of blueberry juice was explored. It was found that blueberries inhibited cell growth in a dose-dependent way. Subsequent to the exposure of SKOV3 cells to 16 mg/ml blueberry, the cell growth rate was reduced by up to 28% compared with the control following the 3-day culture (P=0.018; Fig. 1). Based on these results, the present study used a higher blueberry concentration (16 mg/ml) to treat the OC mouse models (400 mg daily for a 25 g mouse).

Effects of COX-1 and COX-2 on the growth rate of SKOV3 cells. To investigate the effects of COX-1 and COX-2 on SKOV3 cells, the genes were overexpressed by gene transformation or inhibited by RNAi in the cells. Exposure of SKOV3 cells to 16 mg/l blueberry resulted in a reduction of cell concentration detected by MTT when compared with the control (P=0.019; Fig. 1). When COX-1 or COX-2 was inhibited by RNAi in SKOV3 cells and treated with 16 mg/ml blueberry juice, the growth rate of the cells was reduced by up to 50% compared with the growth rate of the control group subsequent to 72 h culture (Fig. 1). By contrast, if COX-1 or COX-2 was overexpressed in SKOV3 cells, the growth rate increased by up to 66% compared with the growth rate of the control group subsequent to 72 h culture (Fig. 1). The increase was inhibited completely when 16 mg/ml blueberry juice was added to the cells.

mRNA level of COX-1 and COX-2 in SKOV3. The mRNA level of COX-1 was significantly affected by blueberry juice and was reduced by up to 50% when 16 mg/ml blueberry juice was added compared with the control without the blueberry treatment (P=0.002; Fig. 2). The mRNA level of COX-1 increased by up to 50% when SKOV3 cells were transfected with COX-1, compared with the control without COX-1 transfection (P=0.002; Fig. 2). The increase was inhibited by the addition of blueberry juice in a dose-dependent way. The mRNA level of COX-1 was reduced to 0 when SKOV3 cells were transfected with COX-1 RNAi (Fig. 2). Neither the overexpression nor gene silencing of COX-2 affected the mRNA level of COX-1 (P=0.235; Fig. 2).
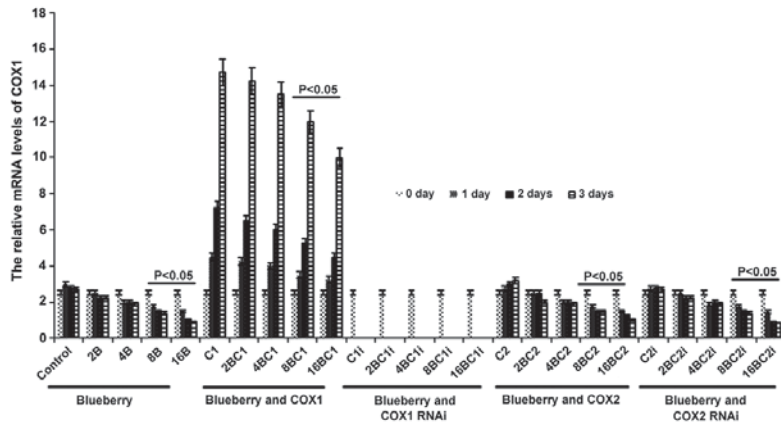
Figure 2. Reverse transcription-qualitative polymerase chain reaction analyses show that blueberries reduce relative mRNA levels of COX-1 in SKOV3 cells. SKOV3 cells were transfected with COX-1, COX-2, COX-1 RNAi or COX-2 RNAi, and treated with 0, 2, 4, 8 or 16 mg/ml blueberry juice. The relative mRNA levels of COX-1 were measured at days 0, 1, 2 and 3. GAPDH was used as an internal control. The data represent the results of 5 independent experiments and are presented as the mean ± standard deviation. P<0.05 compared with 0 µg/ml blueberry subsequent to a 1-, 2- or 3-day culture. COX, cyclooygenase; RNAi, RNA interference; Control, without transfection or blueberry; 2B, 2 mg blueberry; 4B, 4 mg blueberry; 8B, 8 mg blueberry; 16B, 16 mg blueberry; C1, COX-1; 2BC1, 2 mg blueberry and COX1 transfection; 4BC1, 4 mg blueberry and COX1 transfection; 8BC1, 8 mg blueberry and COX1 transfection; 16BC1, 16 mg blueberry and COX1 transfection; C1i, COX-1 RNAi; 2BC1i, 2 mg blueberry and COX1 RNAi; 4BC1i, 4 mg blueberry and COX1 RNAi; 8BC1i, 8 mg blueberry and COX1 RNAi; 16BC1i, 16 mg blueberry and COX1 RNAi; C2, COX-2; 2BC2, 2 mg blueberry and COX2 transfection; 4BC2, 4 mg blueberry and COX2 transfection; 8BC2, 8 mg blueberry and COX2 transfection; 16BC2, 16 mg blueberry and COX2 transfection; C2i, COX-2 RNAi; 2BC2i, 2 mg blueberry and COX2 RNAi; 4BC2i, 4 mg blueberry and COX2 RNAi; 8BC2i, 8 mg blueberry and COX2 RNAi; 16BC2i, 16 mg blueberry and COX2 RNAi.
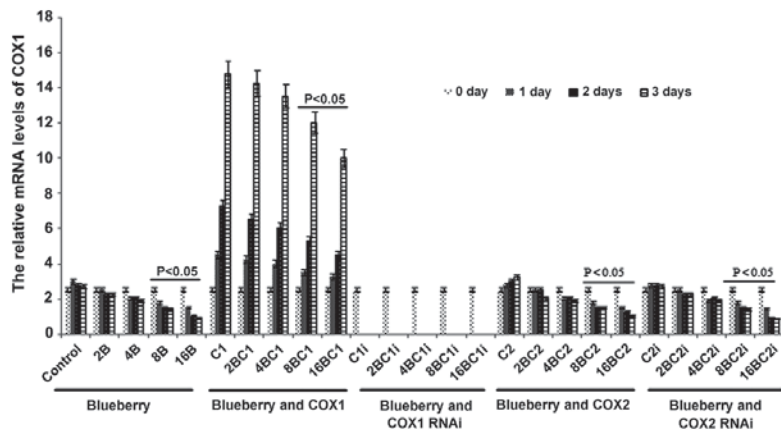

Figure 3. Reverse transcription qualitative-polymerase chain reaction analyses show that blueberries reduce relative mRNA levels of COX-2 in SKOV3 cells. SKOV3 cells were transfected with COX-1, COX-2, COX-1 RNAi or COX-2 RNAi, and treated with 0, 2, 4, 8 or 16 mg/ml blueberry juice. The relative mRNA levels of COX-2 were measured at days 0, 1, 2 and 3. GAPDH was used as an internal control. The data represent the results of 5 independent experiments and are presented as the mean ± standard deviation. P<0.05 compared with 0 µg/ml blueberry subsequent to a 1-, 2- or 3-day culture. COX, cyclooygenase; RNAi, RNA interference; Control, without transfection or blueberry; 2B, 2 mg blueberry; 4B, 4 mg blueberry; 8B, 8 mg blueberry; 16B, 16 mg blueberry; C1, COX-1; 2BC1, 2 mg blueberry and COX1 transfection; 4BC1, 4 mg blueberry and COX1 transfection; 8BC1, 8 mg blueberry and COX1 transfection; 16BC1, 16 mg blueberry and COX1 transfection; C1i, COX-1 RNAi; 2BC1i, 2 mg blueberry and COX1 RNAi; 4BC1i, 4 mg blueberry and COX1 RNAi; 8BC1i, 8 mg blueberry and COX1 RNAi; 16BC1i, 16 mg blueberry and COX1 RNAi; C2, COX-2; 2BC2, 2 mg blueberry and COX2 transfection; 4BC2, 4 mg blueberry and COX2 transfection; 8BC2, 8 mg blueberry and COX2 transfection; 16BC2, 16 mg blueberry and COX2 transfection; C2i, COX-2 RNAi; 2BC2i, 2 mg blueberry and COX2 RNAi; 4BC2i, 4 mg blueberry and COX2 RNAi; 8BC2i, 8 mg blueberry and COX2 RNAi; 16BC2i, 16 mg blueberry and COX2 RNAi.

The mRNA level of COX-2 was significantly affected by blueberry juice, decreasing by up to 50% when 16 mg/ml blueberry was added compared with the control without the blueberry treatment (P=0.008; Fig. 3). The mRNA level of COX-2 also increased by up to 150% when the SKOV3 cells were transfected with COX-2 compared with the control without COX-2 transfection (P=0.002; Fig. 3). The increase was inhibited by the addition of blueberry juice in a dose-dependent way. The mRNA level of COX-2 decreased to 0 when the SKOV3 cells were transfected with COX-2 RNAi (Fig. 3). Neither the overexpression nor gene silencing

of COX-1 affected the mRNA level of COX-2 (P=0.321; Fig. 3).

Levels of COX-1 and COX-2 in SKOV3. The results of the levels of COX-1 and COX-2 in SKOV3 were similar compared with the results from the mRNA levels investigation. The concentration of COX-1 was significantly affected by the blueberry juice, being reduced by up to 50% when 16 mg/ml blueberry juice was added, compared with the control without the blueberry treatment (P=0.003; Fig. 4). The concentration of COX-1 increased by up to 50% when the SKOV3 cells were
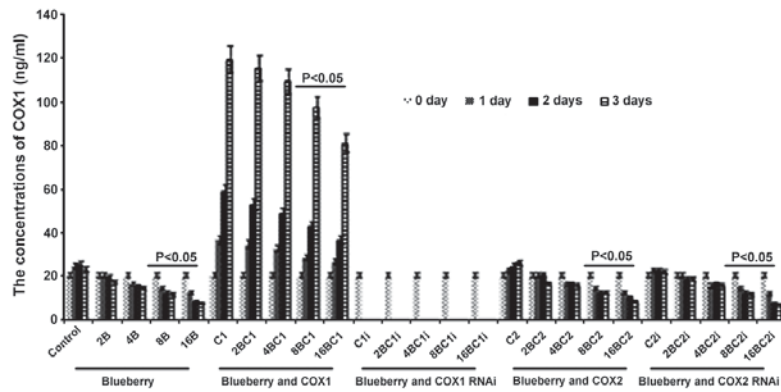
Figure 4. ELISA analyses show that blueberries reduce the protein levels of COX-1 in SKOV3 cells. SKOV3 cells were transfected with COX-1, COX-2, COX-1 RNAi or COX-2 RNAi, and treated with 0, 2, 4, 8 or 16 mg/ml blueberry juice. The concentrations of COX-1 were measured at days 0, 1, 2 and 3. The data represent the results of 5 independent experiments and are presented as the mean ± standard deviation. P<0.05 compared with 0 μg/ml blueberry subsequent to a 1-, 2- or 3-day culture. COX, cyclooxygenase; RNAi, RNA interference; Control, without transfection or blueberry; 2B, 2 mg blueberry; 4B, 4 mg blueberry; 8B, 8 mg blueberry; 16B, 16 mg blueberry; C1, COX-1; 2BC1, 2 mg blueberry and COX1 transfection; 4BC1, 4 mg blueberry and COX1 transfection; 8BC1, 8 mg blueberry and COX1 transfection; 16BC1, 16 mg blueberry and COX1 transfection; C1i, COX-1 RNAi; 2BC1i, 2 mg blueberry and COX1 RNAi; 4BC1i, 4 mg blueberry and COX1 RNAi; 8BC1i, 8 mg blueberry and COX1 RNAi; 16BC1i, 16 mg blueberry and COX1 RNAi; C2, COX-2; 2BC2, 2 mg blueberry and COX2 transfection; 4BC2, 4 mg blueberry and COX2 transfection; 8BC2, 8 mg blueberry and COX2 transfection; 16BC2, 16 mg blueberry and COX2 transfection; C2i, COX-2 RNAi; 2BC2i, 2 mg blueberry and COX2 RNAi; 4BC2i, 4 mg blueberry and COX2 RNAi; 8BC2i, 8 mg blueberry and COX2 RNAi; 16BC2i, 16 mg blueberry and COX2 RNAi.
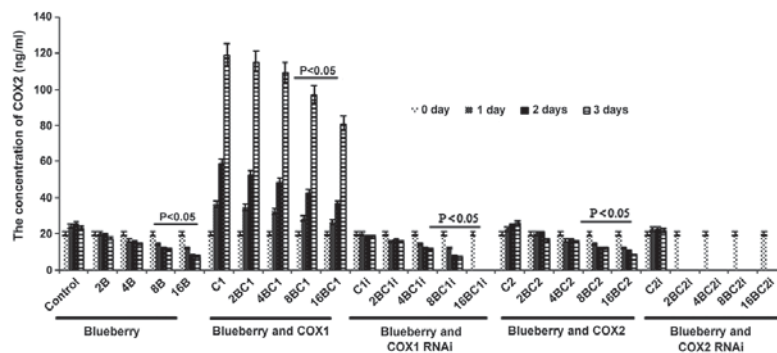

Figure 5. ELISA analyses show that blueberries reduce the protein levels of COX-2 in SKOV3 cells. SKOV3 cells were transfected with COX-1, COX-2, COX-1 RNAi or COX-2 RNAi, and treated with 0, 2, 4, 8 or 16 mg/ml blueberry juice. The concentrations of COX-2 were measured at days 0, 1, 2 and 3. The data represents the results of 5 independent experiments and are presented as the mean ± standard deviation. P<0.05 compared with 0 μg/ml blueberry subsequent to a 1-, 2- or 3-day culture. COX, cyclooxygenase; RNAi, RNA interference; Control, without transfection or blueberry; 2B, 2 mg blueberry; 4B, 4 mg blueberry; 8B, 8 mg blueberry; 16B, 16 mg blueberry; C1, COX-1; 2BC1, 2 mg blueberry and COX1 transfection; 4BC1, 4 mg blueberry and COX1 transfection; 8BC1, 8 mg blueberry and COX1 transfection; 16BC1, 16 mg blueberry and COX1 transfection; C1i, COX-1 RNAi; 2BC1i, 2 mg blueberry and COX1 RNAi; 4BC1i, 4 mg blueberry and COX1 RNAi; 8BC1i, 8 mg blueberry and COX1 RNAi; 16BC1i, 16 mg blueberry and COX1 RNAi; C2, COX-2; 2BC2, 2 mg blueberry and COX2 transfection; 4BC2, 4 mg blueberry and COX2 transfection; 8BC2, 8 mg blueberry and COX2 transfection; 16BC2, 16 mg blueberry and COX2 transfection; C2i, COX-2 RNAi; 2BC2i, 2 mg blueberry and COX2 RNAi; 4BC2i, 4 mg blueberry and COX2 RNAi; 8BC2i, 8 mg blueberry and COX2 RNAi; 16BC2i, 16 mg blueberry and COX2 RNAi.

transfected with COX-1, compared with the control without COX-1 transfection (P=0.002; Fig. 4). The increase was inhibited by the addition of blueberry juice in a dose-dependent way. The concentration of COX-1 decreased to 0 when the SKOV3 cells were transfected with COX-1 RNAi (Fig. 4). Neither the overexpression nor gene silencing of COX-2 affected the concentration of COX-1 (P=0.287; Fig. 4).

Similarly, the concentration of COX-2 was significantly affected by blueberry juice, being reduced by up to 50% when 16 mg/ml blueberry juice was added, compared with the control without the blueberry treatment (P=0.002; Fig. 5). The mRNA level of COX-2 increased by up to 150% when SKOV3 cells were transfected with COX-2, compared with the control without COX-2 transfection (P=0.003; Fig. 5). The increase was inhibited by the addition of blueberry juice in a

dose-dependent way. The concentration of COX-2 decreased to 0 when the SKOV3 cells were transfected with COX-2 RNAi (Fig. 5). Neither the overexpression nor gene silencing of COX-1 affected the concentration of COX-2 (P=0.329; Fig. 3).

The aforementioned results suggest that blueberries reduce the concentration of COX-1 and COX-2 in a dose-dependent way. Blueberries inhibit the growth rate of SKOV3 by decreasing the levels of COX-1 and COX-2, while COX-1 and COX-2 promote the growth of SKOV3.

Effects of blueberry on tumor volume. Prior to the blueberry treatment, the volumes of the OC tumors were similar between groups. Subsequent to the blueberry treatment, the volumes of OC in the 500 mg group significantly decreased by 40% compared with the group that received no
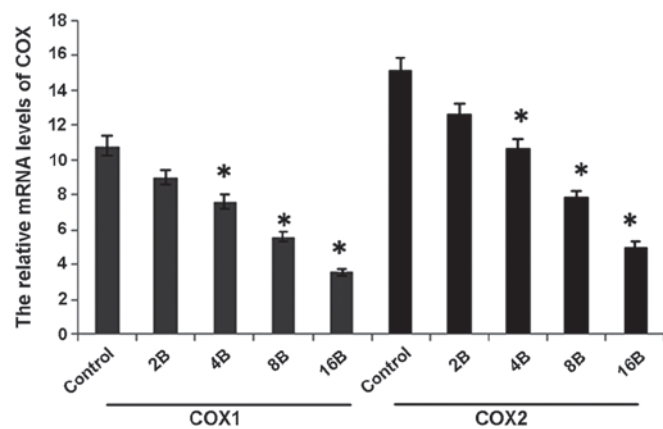
Figure 6. Reverse transcription quantitative-polymerase chain reaction analyses show that blueberries reduce relative mRNA levels of COX-1 and COX-2 of ovarian cancer in a mouse model. The mice were treated with 0, 2, 4, 8 or 16 mg/ml blueberry juice. The relative mRNA levels of COX-1 and COX-2 were measured subsequent to a 14-day culture. GAPDH was used as an internal control. The data represent the results of 5 independent experiments and are presented as the mean ± standard deviation. *P<0.05 compared with 0 µg/ml blueberry. COX, cyclooxygenase; Control, without transfection or blueberry; 2B, 2 mg blueberry; 4B, 4 mg blueberry; 8B, 8 mg blueberry; 16B, 16 mg blueberry.
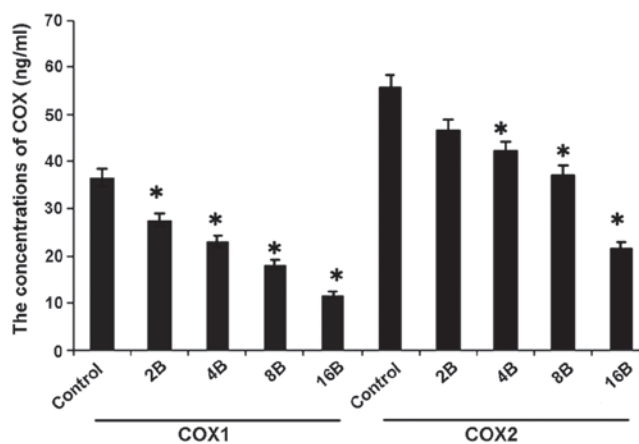

Figure 7. ELISA analyses show that blueberries reduce protein levels of COX-1 and COX-2 of ovarian cancer in a mouse model. The mice were treated with 0, 2, 4, 8 or 16 mg/ml blueberry juice. The protein levels of COX-1 and COX-2 were measured subsequent to a 14-day culture. The data represent the results of 5 independent experiments and are presented as the mean ± standard deviation. *P<0.05 compared with 0 µg/ml blueberry. COX, cyclooxygenase, Control, without transfection or blueberry; 2B, 2 mg blueberry; 4B, 4 mg blueberry; 8B, 8 mg blueberry; 16B, 16 mg blueberry.

blueberry treatment (P=0.0008). The high-efficiency inhibitory concentrations of blueberry were between 8 and 16 mg. The weights of the OC tumors were 4.82±0.41, 4.25±0.35, 3.16±0.23 and 2.47±0.26 g in blank, 0, 100, 200 and 400 mg blueberry groups, respectively, yielding growth inhibitions of 12.5, 35.4, and 49.4%. From the aforementioned results, the 400 mg blueberry group showed significant inhibitory results for OC (P=0.014).

Blueberry juice affects the mRNA levels of COX-1 and COX-2 of OC in the mouse model. The present study investigated the effect of blueberries on the mRNA levels of COX-1 and COX-2 of OC in a mouse model, which are biomarkers of OC (24,28). The mRNA levels of COX-1 and COX-2 of OC in the mice were the highest in the control group compared with those in the models treated with blueberry juice (P=0.006; Fig. 6). Comparatively, the levels of COX-1 and COX-2 significantly decreased by up to 63.6 and 68.7%, respectively, when the mice were treated with 400 mg blueberry juice daily compared with those from the control without the addition of blueberry juice (P=0.002; Fig. 6). The results suggested that blueberry juice significantly affects the mRNA levels of COX-1 and COX-2 of OC in the mouse model studied.

Blueberry juice affects the levels of COX-1 and COX-2 of OC in the mouse model. In concordance with the mRNA results, ELISA analysis showed higher levels of COX-1 and COX-2 of OC in mice in the control group when compared with the groups treated with blueberry juice (P=0.012; Fig. 7). Comparatively, the levels of COX-1 and COX-2 significantly decreased by up to 57.1 and 54.5%, respectively, when the mice were treated with 400 mg blueberry juice daily compared with the protein levels of the control without the addition of blueberry juice (P=0.006; Fig. 7). The aforementioned results suggested that blueberry juice significantly affects the levels of COX-1 and COX-2 of OC in the mouse model studied.

Discussion

OC is a common cause of female mortality worldwide and blueberry therapy has been identified to be effective in the treatment of various types of carcinoma (21,22,29). The theoretical benefit of blueberry juice as a salvage therapy is associated with the anti-inflammation capacity (30,31) and ability to prevent the progression of various types of cancer (32,33). However, the molecular mechanism of the inhibition of OC by blueberries remains unclear. Therefore, the present study aimed to address the issue, and revealed a significant result with respect to the use of blueberry juice for the treatment of OC in the BALB/c nude mouse model. Based on the aforementioned information, the present study firstly reported the molecular mechanisms for the inhibition of OC by blueberry, revealing that a suitable dosage of blueberry juice decreases the expression of COX-1 and COX-2, which are 2 biomarkers for the development of OC. The aforementioned findings suggest that blueberry juice decreases the levels of COX-1 and COX-2, and inhibits the progression of OC. Furthermore, detecting the levels of COX-1 and COX-2 aid in the prediction of patients at risk for OC, as reported in previous studies (24,34). Blueberry juice therapy may therefore provide a non-pharmaceutical treatment for patients with OC.

Regarding the promotion of the growth of SKOV3 by COX-1 and COX-2, the issue that growth rate may be affected by other molecules must be considered. Therefore, the present study investigated the overexpression and gene silencing of COX-1 and COX-2, revealing that when COX-1 and COX-2 were overexpressed without blueberry treatment, the growth rate of SKOV3 reached the highest level of the present study. By contrast, when COX-1 and COX-2 were silenced by RNAi and treated with blueberry juice at a high concentration, the growth rate of SKOV3 reached the lowest level of the present study (Fig. 1). Furthermore, blueberry juice reduces the levels of COX-1 and COX-2 in a dose-dependent way (Figs. 2-5). The aforementioned results suggest that COX-1 and COX-2

promote the growth of OC while blueberry juice inhibits the development of OC by downregulating the levels of COX-1 and COX-2.

There were certain limitations of the present study. For example, blueberry juice possesses a number of different components, which were not separated or purified to identify the more effective agents for the treatment of OC. The functions of blueberries are not unique and more functions require exploration; a limitation of the present study is that the precise mechanism of blueberries with respect to the downregulation of the levels of COX-1 and COX-2 remains unknown. The components of blueberry juice should be analyzed in detail, which may offer information to understand the exact molecular mechanisms for the role of blueberry juice in the therapy of OC.

The present study revealed that the efficacy of using blueberries to treat OC is significant. To make full use of blueberries, all associated molecular mechanisms should be investigated to maximize the potential benefit of blueberries and minimize the risk of side effects. The present study demonstrated that the concentration at which blueberries exhibit significant inhibition is 16 mg/ml for OC cells. At this concentration, blueberries effectively treated the OC models. The results of the present study provide information for subsequent clinical trials and may be beneficial to utilize blueberries, effectively and safely, as a non-pharmaceutical OC therapy. Considering the effectiveness and safety of blueberries, a larger sample size and long-term follow-up test in a larger sample of mice is recommended.

In conclusion, blueberries have been demonstrated to be effective and safe in controlling the size of OC in a dose-dependent way. Blueberries inhibit the proliferation of OC cells by downregulating the levels of COX-1 and COX-2. The present study established animal models of OC by the injection of SKOV3 cells into nude mice. Blueberry (400 mg daily) consumption decreased tumor size significantly in the mice with OC compared with the controls without blueberry treatment ($P<0.05$). The results of the present study suggest that blueberries should be developed as a potential non-pharmaceutical therapy for OC.

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