

Anthocyanin-rich blackcurrant extract inhibits proliferation of the MCF10A healthy human breast epithelial cell line through induction of G0/G1 arrest and apoptosis

NAOKI NANASHIMA¹, KAYO HORIE¹, MITSURU CHIBA¹,
MANABU NAKANO¹, HAYATO MAEDA² and TOSHIYA NAKAMURA¹

¹Department of Biomedical Sciences, Hirosaki University Graduate School of Health Sciences, Hirosaki, Aomori 036-8564;

²Faculty of Agriculture and Life Science, Hirosaki University, Hirosaki, Aomori 036-8561, Japan

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Abstract. Blackcurrants (*Ribes nigrum* L., Grossulariaceae) possess a high content of anthocyanin polyphenols, which have been demonstrated to exhibit beneficial effects on health due to their antioxidant and anticarcinogenic properties. The present study investigated novel functions of anthocyanin-rich blackcurrant extracts (BCEs) in a healthy mammary epithelial cell line, MCF10A. The percentages of viable cells were 85, 75, 53 and 31% following exposure to 50, 100, 200 and 400 μ g/ml BCE, respectively. The half-maximal response concentration of BCE was 237.7 μ g/ml. Microarray and Ingenuity® Pathway Analysis demonstrated that BCE downregulated cell cycle signaling, including upstream genes with mitotic roles such as polo-like kinase signaling. BCE increased the number of cells in the G0/G1 phase and decreased the number of cells in the S and G2/M phases. Alkaline comet assays demonstrated that 50 and 100 μ g/ml BCE induced DNA damage in a dose-dependent manner. Cultures treated with 0, 50, and 100 μ g/ml BCE contained 4.6, 13.4 and 16.0% apoptotic cells, respectively. As compared with the untreated cultures (1.9%), the number of necrotic cells increased in the 100 μ g/ml BCE-treated cultures (from 1.9 to 4.3%) but not in the 50 μ g/ml BCE-treated cultures. Reverse transcription-quantitative polymerase chain reaction analysis demonstrated that BCE reduced mRNA expression of the genomic caretaker lysine-specific demethylase 5B (KDM5B).

The results suggested that blackcurrant anthocyanins may act as cell arrest and death inducers via KDM5B downregulation in healthy breast cells.

Introduction

Blackcurrants (*Ribes nigrum* L., Grossulariaceae) contain a number of biochemical constituents, including polyphenols, polyunsaturated fatty acids, organic acids and phenolic acids (1-4). Blackcurrant flavonoids are a group of polyphenolic compounds that include anthocyanins and flavonols. Anthocyanins are natural plant pigments that are widely distributed among flowers, fruits and vegetables. Blackcurrants are reported to contain four anthocyanins: Delphinidin-3-glucoside; delphinidin-3-rutinoside; cyanidin-3-glucoside; and cyanidin-3-rutinoside. Delphinidin-3-rutinoside and cyanidin-3-rutinoside are specific blackcurrant anthocyanins (2). These blackcurrant constituents have been associated with certain health benefits, including improved blood flow (5), decreased inflammatory marker levels (6) and protection against DNA damage induced by hydrogen peroxide (7).

Previous studies have demonstrated that blackcurrant phytochemicals exhibit anti-breast cancer effects. For example, blackcurrant extract (BCE) or juice has been demonstrated to inhibit breast cancer cell proliferation (8-10). However, the effects of BCEs on normal human breast epithelial cells have not been reported.

The objective of the present study was to investigate whether an anthocyanin-rich BCE confers novel health benefits to healthy breast epithelial cells. MCF10A healthy human breast epithelial cells were exposed to BCE, and microarray and Ingenuity® Pathway Analysis was performed to assess alterations in gene expression. The regulation of the cell cycle and the impact on cell viability was evaluated using fluorescence-activated cell sorting. DNA damage and cell death was examined by detecting apoptotic and necrotic cells using a comet assay. mRNA expression of the DNA stability regulator lysine-specific demethylase 5B (KDM5B) (11,12) was investigated using the reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Correspondence to: Dr Naoki Nanashima, Department of Biomedical Sciences, Hirosaki University Graduate School of Health Sciences, 66-1 Hon, Hirosaki, Aomori 036-8564, Japan
E-mail: nnaoki@hirosaki-u.ac.jp

Abbreviations: AURKA, aurora kinase A; BCE, blackcurrant extract; KDM5B, lysine-specific demethylase 5B; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: apoptosis, aurora kinase A, blackcurrant, DNA damage, lysine-specific demethylase 5B

Materials and methods

Materials and cell culture. The BCE powder, CaNZac-35, was purchased from Koyo Mercantile Co., Ltd. (Tokyo, Japan). The BCR powder contained high concentrations of polyphenols (37.6 g polyphenol/100 g BCE) and anthocyanins (38.0 g anthocyanin/100 g BCE) (13). MCF10A breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in a Mammary Epithelial Cell Growth Medium kit (PromoCell GmbH, Heidelberg, Germany). The culture experiments were conducted at 37°C in a humidified 5% CO₂ incubator.

Cell viability assay. MCF10A cells were seeded in 96-well plates at 5,000 cells/well and cultured overnight. Cells were then incubated with 0, 10, 25, 50, 100, 200 and 400 µg/ml BCE for 24 h, and observed under a fluorescence microscope at x200 magnification (FSX100; Olympus Corporation, Tokyo, Japan). Cell viability was determined using the PrestoBlue cell viability reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. PrestoBlue reagent was added to the cells and cells were incubated for 6 h at 37°C. Absorbance was measured on a Benchmark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 570 nm. The absorbance was assumed to be directly proportional to the number of cells. BCE concentration-cell growth (dose effect) curves were drawn for each treatment and approximate half-maximal response concentration (EC₅₀) values were determined using Excel software (version 2013; Microsoft Corporation, Redmond, WA, USA).

Microarray gene expression profiling. MCF10A cells were seeded in 21-cm² culture dishes in mammary epithelial cell growth medium and grown under the conditions described above until confluent, at which point the medium was replaced with fresh medium with or without BCE (50 µg/ml). After 24 h incubation, the cells were washed twice with PBS and total RNA was extracted using the RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA). Total RNA (100 ng) was used to produce cyanine 3-CTP-labeled cRNA using a low input quick amp labeling kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Labeled and fragmented cRNA was hybridized to a SurePrint G3 Human Gene Expression microarray (8x60K; version 2; Agilent Technologies, Inc.). Labeling, hybridization, image scanning and data analysis was performed at Bio Matrix Research, Inc. (Chiba, Japan). The MCF10A microarray dataset is available at www.ncbi.nlm.nih.gov/geo under accession code GSE58304.

Genes with ≥2-fold upregulation following exposure of MCF10A cells to 50 µg/ml BCE were analyzed using Ingenuity® Pathway Analysis software (version 18030641; Qiagen, Inc.).

Cell cycle analysis. Cell cycle analysis was performed as previously described (13,14). MCF10A cells were seeded in 21-cm² culture dishes at 5x10⁵ cells/well and cultured overnight. The medium was then replaced with fresh medium with or without BCE (0, 50 or 100 µg/ml). The cells were cultured for 24 h prior to DNA staining with propidium iodide at

room temperature for 2 h. Cell cycle analysis was performed by fluorescence-activated cell sorting, which was conducted using a Cell Cycle Phase Determination kit (Cayman Chemical Company, Ann Arbor, MI, USA) and a Cytomics FC500 flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Data were analyzed using CXP analysis software (version 2.0; Beckman Coulter, Inc.).

Alkali comet assay. A Trevigen Comet Assay™ kit (Trevigen, Gaithersburg, MD, USA) was used to assess DNA strand breaks. MCF10A cells were treated with BCE or H₂O₂ as a positive control for 24 h and resuspended in ice-cold PBS at a density of 1x10⁵ cells/ml. A 50-µl aliquot of cell suspension (containing 1x10⁵ cells/ml) was mixed with 500 µl 1% low-melting agarose maintained at 37°C. A total 50 µl mixture was immediately obtained and evenly spread onto the comet slides. To accelerate gelling of the agarose disc, the slides were incubated at 4°C in the dark for 10 min. The slides were subsequently transferred to a prechilled lysis solution and incubated for 60 min at 4°C. The slides were then transferred to an alkali unwinding solution [200 mM NaOH, 1 mM EDTA (pH>13.0)] and incubated at room temperature for 20 min in the dark to perform denaturation. Subsequently, the slides were transferred to a prechilled alkaline electrophoresis solution and electrophoresed at 21 V for 30 min followed by washing with distilled water. The slides were then immersed in ice-cold 70% ethanol at room temperature for 5 min and air-dried. The slides were incubated with 100 µl SYBR Green I dye [Thermo Fisher Scientific, Inc.; in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA buffer] for 5 min at 4°C to stain the DNA and were immediately analyzed using an FSX100 fluorescence microscope. The images were captured at x100 magnification. Quantitative analysis of tail length was performed using the Comet Assay IV image analysis system (Perceptive Instruments Ltd., Bury St Edmunds, UK).

Cell death detection. MCF10A cells were seeded in 9-cm² culture dishes and cultured overnight. The medium was then replaced with fresh medium with or without BCE (0, 50 or 100 µg/ml). Apoptosis and necrosis was detected using a Cell Meter™ Apoptotic and Necrotic Detection kit (AAT Bioquest, Inc., Sunnyvale, CA, USA). The cells were observed with an FSX100 fluorescence microscope. The images were captured at x42 magnification. Quantitative analysis of apoptotic and necrotic cells was performed using FSX-BSW software (version 03.01; Olympus Corporation).

RT-qPCR. MCF10A cells were seeded in 9-cm² culture dishes and cultured as described above until confluent. The used medium was then replaced with fresh medium with or without BCE (0, 25 or 50 µg/ml). The cells were incubated for 24 h and washed twice with PBS. Total RNA was extracted using the RNeasy mini kit (Qiagen, Inc.). cDNA was reverse-transcribed from total RNA (0.5 µg) using the Omniscript RT kit (Qiagen, Inc.). A MiniOpticon Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) and GoTaq Green Master Mix (Promega Corporation, Madison, WI, USA) were used for the quantification of specific mRNA. PCRs were denatured at 92°C for 2 min, and amplified at 92°C for 30 sec, 58°C for 30 sec and 72°C for 30 sec, for 40 cycles. Transcript levels were normalized to that of GAPDH cDNA. The primers used

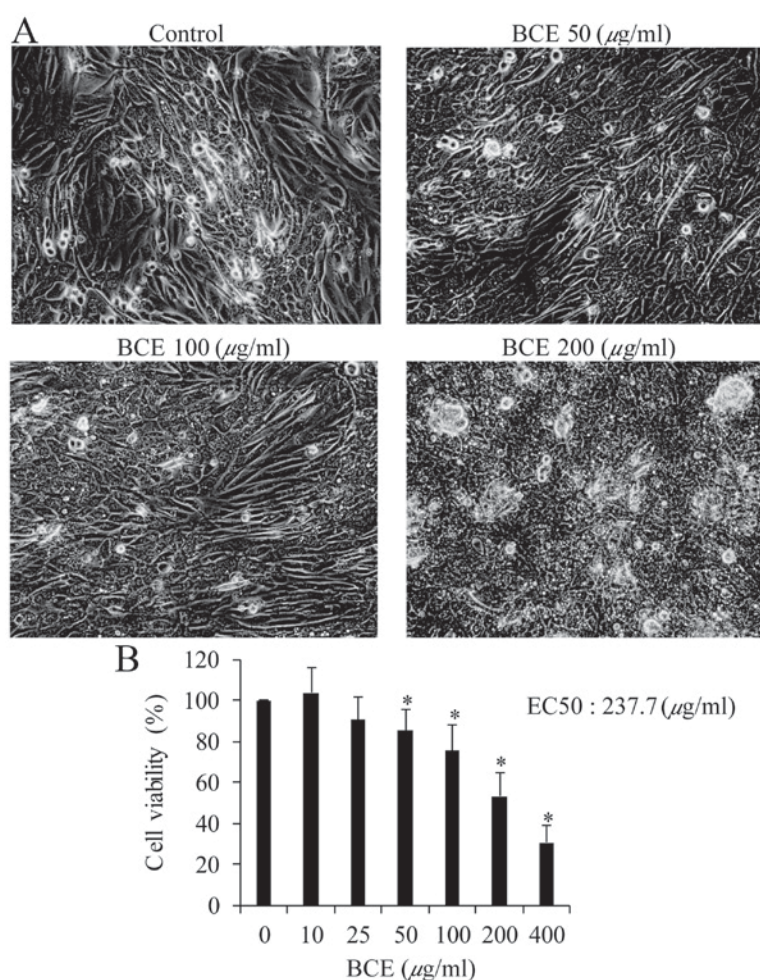


Figure 1. MCF10A cell morphology and cell viability following exposure to BCE. (A) Light microscopy images of MCF10A cells treated with various concentrations of BCE (magnification, x200). (B) Viability of MCF10A cells treated with BCE. Data are presented as the mean \pm standard error of the mean from at least three independent experiments. * $P < 0.01$ vs. the untreated control cells. BCE, blackcurrant extract; EC₅₀, half maximal response concentration.

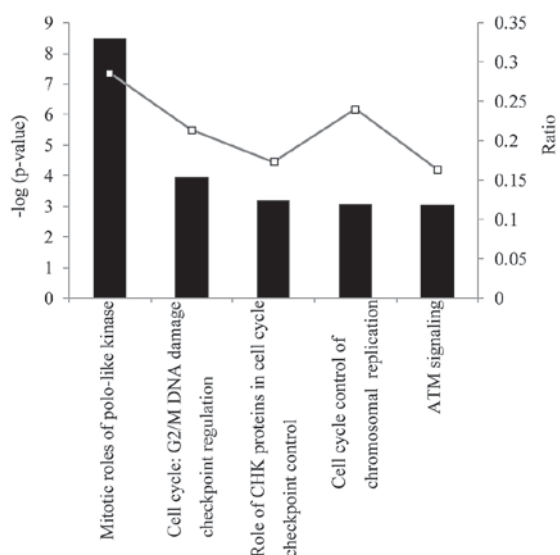


Figure 2. Canonical pathways in the MCF10A cells. The bar graph (ratio) and the line graph (P-value) are representative of results from the Ingenuity® Pathway Analysis of microarray data from MCF10A breast epithelial cells treated with 50 $\mu\text{g/ml}$ blackcurrant extract. The five most regulated canonical pathways in MCF10A are presented. The bar graph represents the number of significantly expressed genes in each canonical pathway over the total number of genes involved in that pathway. CHK, checkpoint kinase; ATM, ataxia telangiectasia mutated.

were as follows (5'-3'): KDM5B forward, ATTGCCTCAAAG GAATTTGGCAGTG and reverse, CATCACTGGCATGTT GTTCAAATTC; GAPDH forward, AGAAGGCTGGGGCTC ATTTG and reverse, AGGGGCCATCCACAGTCTTC. PCR specificity was confirmed by melting curve analysis. Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (15).

Statistical analysis. Results are presented as the mean \pm standard error of the mean of at least three independent experiments. Statistical significance was determined using one-way analysis of variance followed by Tukey's test. SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BCE decreases MCF10A cell viability. To investigate BCE toxicity to MCF10A cells, viability was determined using the PrestoBlue assay. Morphological alterations were observed in cells exposed to 200 $\mu\text{g/ml}$ BCE but not in cells exposed to the lower concentrations of 50 and 100 $\mu\text{g/ml}$ (Fig. 1A). The percentages of cells that remained viable following exposure to 50, 100, 200 and 400 $\mu\text{g/ml}$ BCE were 85, 75, 53 and 31%,

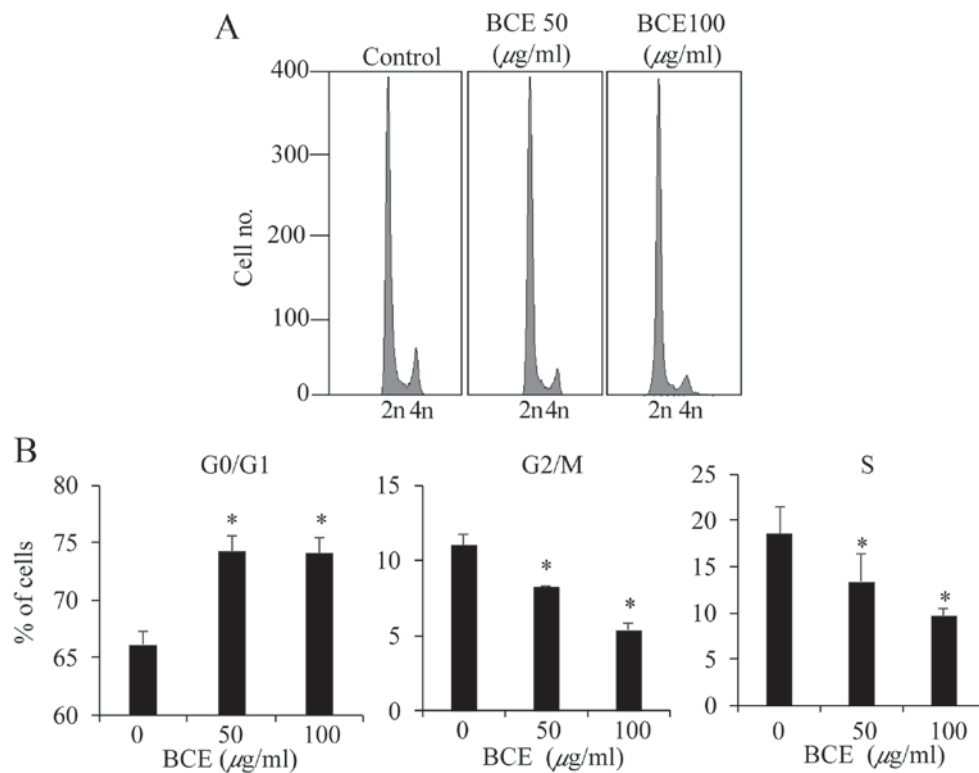


Figure 3. Cell cycle analysis in MCF10A cells treated with BCE for 24 h prior to DNA staining and fluorescence-activated cell sorting. (A) Cell cycle plots. (B) The percentages of cells in the G0/G1, G2/M and S phases. Data were analyzed using MultiCycle AV software and represent the mean \pm standard error of the mean from three independent experiments. * $P < 0.01$ vs. the untreated control cells. BCE, blackcurrant extract.

respectively (Fig. 1B). There was no significant alteration in the viability of cells exposed to 10 or 25 µg/ml BCE.

Ingenuity Pathway Analysis. To investigate the impact of BCE on MCF10A cells, gene expression in the cells prior to and following BCE treatment was compared using microarrays. Ingenuity Pathway Analysis was used to investigate the functional associations between sets of genes with modified expression levels. Given that 50 µg/ml BCE did not affect MCF10A cell morphology and the EC_{50} value of BCE was 237.7 µg/ml (Fig. 1B), a concentration of 50 µg/ml was selected for microarray analysis. A total of 147 significant canonical pathways were identified ($P < 0.05$), the majority of which were associated with cell cycle signaling functions. The primary canonical pathways were 'mitotic roles of polo-like kinase', 'G2/M DNA damage checkpoint regulation', 'the role of checkpoint kinase proteins in cell cycle', 'cell cycle control of chromosomal replication checkpoint control' and 'ataxia telangiectasia mutated signaling' (Fig. 2). Detailed gene expression alterations in each significantly affected canonical pathway are presented in Table I. Mitotic roles of polo-like kinase-related genes were affected the most. Certain genes were common genes altered between mitotic roles of polo-like kinase and other canonical pathways (G2/M DNA damage checkpoint regulation, role of checkpoint kinase proteins in cell cycle or ataxia telangiectasia mutated signaling) (Table I). These results indicate that BCE is associated with significantly downregulated expression levels of 'mitotic roles of Polo-like kinase' and 'cell cycle control of chromosomal replication checkpoint control' genes.

Cell cycle analysis. MCF10A cells were treated with BCE (0, 50 and 100 µg/ml) for 24 h prior to cell cycle analysis. Treatment with BCE significantly increased the proportion of G0/G1 phase cells, and decreased the proportions of G2/M phase and S phase cells (Fig. 3). These results indicate that BCE inhibits cell cycle progression by inducing G0/G1 arrest in MCF10A cells.

BCE-induced DNA damage. As DNA damage is an early event of cell cycle arrest, it was further examined whether BCE induces DNA damage in MCF10A cells. As presented in Fig. 4A and B, 50 and 100 µg/ml BCE induced DNA damage in a dose-dependent manner as measured by alkaline comet assay. The comet tail lengths of 0, 50, 100 µg/ml BCE- and H_2O_2 -treated MCF10A cells were 11.4, 15.9, 22.9, and 41.6 µm, respectively. These results suggest that BCE caused significant DNA damage.

Induction of apoptosis and necrosis. As BCE decreased cell viability and induced G0/G1 phase arrest and DNA damage in MCF10A, it was hypothesized that BCE may contribute to cell death. To examine this possibility, the number of apoptotic and necrotic MCF10A cells were counted following treatment with BCE. The cultures treated with 0, 50 and 100 µg/ml BCE contained 4.6, 13.4 and 16.0% apoptotic cells, respectively (Fig. 4C and D). As compared with the untreated cells (1.9%), the percentage of necrotic cells increased in the 100 µg/ml BCE-treated culture (4.3%) but not in the 50 µg/ml BCE-treated culture (Fig. 4C and D). These results indicate that BCE acts as an apoptotic inducer in MCF10A cells.

Table I. Details of altered gene expression in canonical pathways.

Canonical pathway	Gene symbol	Gene name	Fold-change
Mitotic roles of polo-like kinase related genes	AURKA	Aurora kinase A	0.46
	CCNB1	Cyclin B1	0.40
	CCNB2	Cyclin B2	0.37
	CDC20	Cell division cycle 20	0.37
	CDC25A	Cell division cycle 25A	0.44
	CDC25C	Cell division cycle 25C	0.44
	CDK1	Cyclin-dependent kinase 1	0.43
	CHEK2	Checkpoint kinase 2	0.48
	ESPL1	Extra spindle pole bodies homolog 1	0.45
	FBXO5	F-box protein 5	0.43
	KIF11	Kinesin family member 11	0.48
	KIF23	Kinesin family member 23	0.41
	PKMYT1	Protein kinase, membrane associated tyrosine/threonine 1	0.42
	PLK1	Polo-like kinase 1	0.42
	PLK4	Polo-like kinase 4	0.41
	PRC1	Protein regulator of cytokinesis 1	0.39
Cell cycle: G2/M DNA damage checkpoint regulation	CCNB1	Cyclin B1	0.40
	CCNB2	Cyclin B2	0.37
	CDC25C	Cell division cycle 25C	0.44
	CDK1	Cyclin-dependent kinase 1	0.43
	CHEK2	Checkpoint kinase 2	0.48
	CKS2	CDC28 protein kinase regulatory subunit 2	0.49
	PKMYT1	Protein kinase, membrane associated 1 tyrosine/threonine	0.42
	PLK1	Polo-like kinase 1	0.42
	TOP2A	Topoisomerase (DNA) II α	0.46
Role of CHK proteins in cell cycle checkpoint control	CDC25A	Cell division cycle 25A	0.44
	CDC25C	Cell division cycle 25C	0.44
	CDK1	Cyclin-dependent kinase 1	0.43
	CHEK2	Checkpoint kinase 2	0.48
	CLSPN	Claspin	0.50
	PLK1	Polo-like kinase 1	0.42
	RFC3	Replication factor C subunit 3	0.37
	SLC19A1	Solute carrier family 19 (folate transporter), member 1	0.48
Cell cycle control of chromosomal replication	CDC45	Cell division cycle 45	0.38
	CDT1	Chromatin licensing and DNA replication factor 1	0.43
	CHEK2	Checkpoint kinase 2	0.48
	MCM7	Minichromosome maintenance complex component 7	0.48
	ORC1	Origin recognition complex subunit 1	0.31
	ORC6	Origin recognition complex subunit 6	0.37
Ataxia telangiectasia mutated signaling	BLM	Bloom syndrome, recQ helicase-like	0.44
	CCNB1	Cyclin B1	0.40
	CCNB2	Cyclin B2	0.37
	CDC25A	Cell division cycle 25A	0.44
	CDC25C	Cell division cycle 25C	0.44
	CDK1	Cyclin-dependent kinase 1	0.43
	CHEK2	Checkpoint kinase 2	0.48
	RAD51	RAD51 recombinase	0.50
	TP73	Tumor protein p73	15.20

BCE decreases KDM5B expression. As KDM5B is known to be a DNA stability regulator with high expression in breast cancer cells, its gene expression was investigated by RT-qPCR. Treatment with BCE decreased KDM5B expression in a

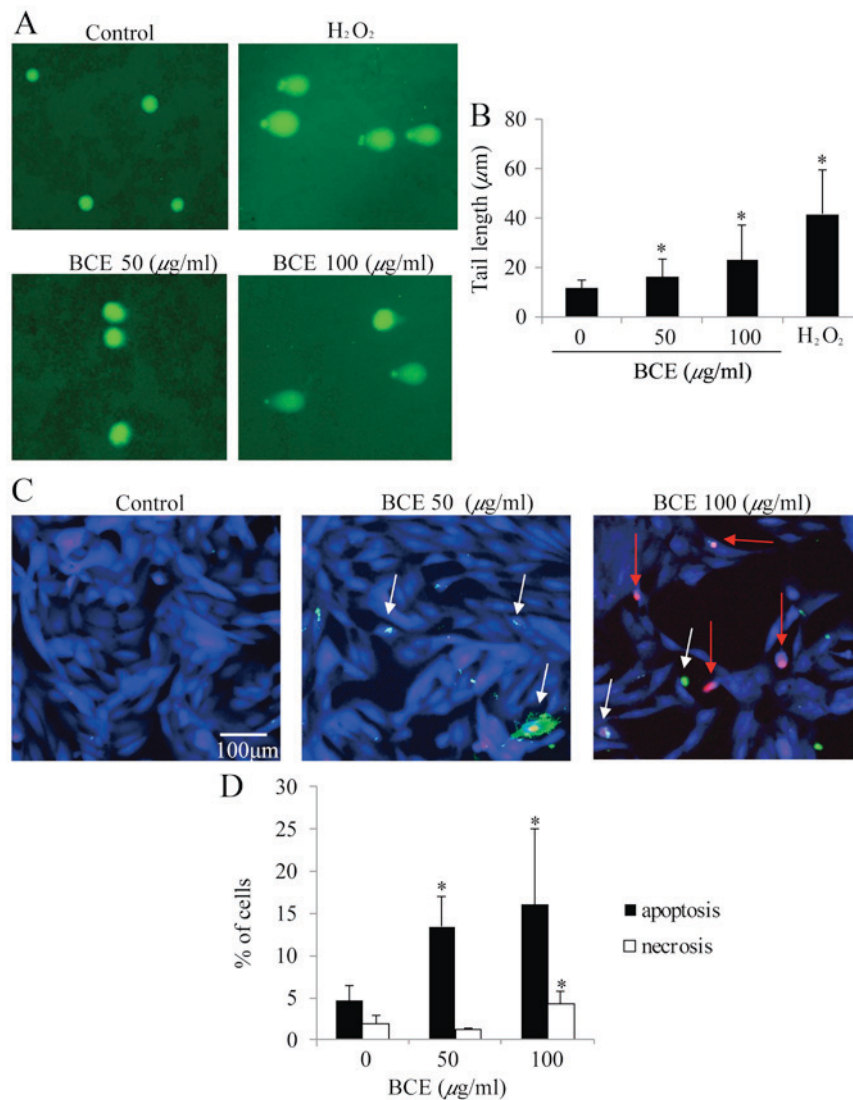


Figure 4. Analysis of DNA damage and cell death in breast epithelial cells treated with BCE. (A) Images of cells treated with BCE for 24 h (with 100 μ M H₂O₂ as positive control). DNA damage was determined using an alkaline comet assay. (B) Quantitative analysis of tail length by Comet Assay IV software. Tail length indicates DNA damage. (C) Images of apoptotic cells (white arrows) and necrotic cells (red arrows). Scale bar, 100 μ m. (D) Percentage of apoptotic and necrotic cells in MCF10A cells treated with BCE. Data represent the mean \pm standard error of the mean from at least three independent experiments. * $P < 0.01$ vs. the untreated control cells. BCE, blackcurrant extract.

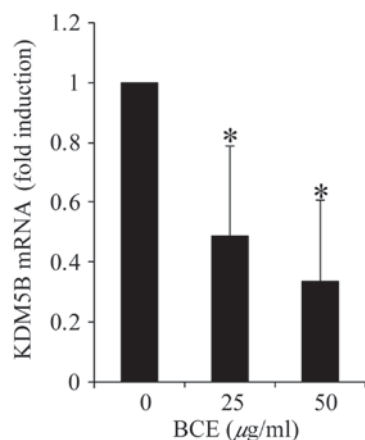


Figure 5. Effect of BCE on the mRNA levels of KDM5B. MCF10A cells were treated with BCE for 24 h. mRNA levels of KDM5B were measured using the reverse transcription-quantitative polymerase chain reaction. Data represent the mean \pm standard error of the mean from at least three independent experiments. * $P < 0.01$ vs. the untreated control cells. BCE, blackcurrant extract; KDM5B, lysine-specific demethylase 5B.

dose-dependent manner (Fig. 5). These results indicate that BCE induces KDM5B downregulation.

Discussion

In addition to the known health benefits of blackcurrants, the present study investigated a novel function of anthocyanin-rich BCE in the MCF10A healthy mammary epithelial cell line. The present study demonstrated that exposure of MCF10A cells to BCE reduces the expression of genes involved in cell signaling pathways, including the mitotic roles of polo-like kinase signaling and associated genes, and induces G0/G1 arrest and cell death. Although canonical pathway analysis demonstrated that BCE reduced certain signaling pathways, certain genes were common among pathways. The important dysregulated signaling pathways appeared to be the 'mitotic roles of polo-like kinase' and 'cell cycle control of chromosomal replication checkpoint control' pathways. Fluorescence-activated cell sorting analysis revealed that cell cycle arrest reduced M

and S phase cells, and the comet assay revealed DNA damage following exposure to 50 or 100 $\mu\text{g/ml}$ BCE. Previous studies have demonstrated that polyphenols induce DNA damage, and G2/M or G0/G1 cell cycle arrest (16-19).

BCE has been demonstrated to exert an anti-proliferative effect on HT29 colon cancer cells, possibly through the suppression of the cyclin-dependent kinase inhibitor 1 (p21^{WAF1}) signaling pathway (20) and a potent cytotoxic effect against hepatocellular carcinoma (21). However, in the present study, microarray and Ingenuity Pathway Analysis did not indicate suppression of the p21^{WAF1} signaling pathway (data not shown).

Microarray and Ingenuity Pathway Analysis revealed a reduction in polo-like kinase signaling and associated genes. It is known that almost every step of cell division, from entry into mitosis to cytokinesis, is regulated by protein phosphorylation by certain kinases, including aurora kinase A (AURKA), AURKB, the cyclin-dependent kinase/cyclin B complex and polo-kinase 1 (PLK1) (22,23). As the AURKs are frequently overexpressed in cancer cells, it is hypothesized that AURKA may cause the progression of gene instability observed in cancer. Furthermore, AURKA and PLK1 cross-talk during mitotic entry and spindle assembly (24). Therefore, inhibitors of AURK activity may have the potential to be used as novel anticancer agents. As BCE suppressed AURKA and PLK1 expression, the present study may lead to the development of a treatment for the prevention of breast cancer.

Phytoestrogens are chemical compounds synthesized by plants and are obtained naturally in a wide range of foods, and can have estrogenic effects (25-28). The authors previously reported that low-density blackcurrant anthocyanins and BCE exhibit phytoestrogenic activity mediated via estrogen receptor α signaling (13). As certain phytoestrogens were demonstrated to inhibit breast cell proliferation and induce apoptosis (29,30), it was hypothesized that BCE may be effective for the prevention of the breast cancer. Anthocyanins or polyphenols cause DNA damage and are known to cause apoptosis, by activation of the ataxia telangiectasia-mutated signaling gene and induction of p53 and p21 (31-33). However, in the present study, ataxia telangiectasia-mutated signaling was not activated and expression of the key regulator of DNA stability, KDM5B, was decreased. These results are different from previous reports.

KDM5B with methylation H3K4 is required for mammary gland development and serves an important role in the proliferative capacity of breast cancer cells (34,35). The interaction of cyclin B1 and cyclin-dependent kinase 1 is essential for control of the cell cycle at the G2/M phase. Conversely, KDM5B has been reported to possibly be involved in the regulation of the G2/M checkpoint and late M phase of the cell cycle (36-39). Furthermore, interference of KDM5B was demonstrated to increase the accumulation of G1 phase MCF7 breast cancer cells (40). As treatment with BCE reduces KDM5B expression, and induces DNA damage, G0/G1 cell cycle arrest and apoptosis in MCF10A cells, BCE may prove to be useful as a breast cancer preventative food.

In conclusion, the present study demonstrated that anthocyanin-rich BCE induces G0/G1 cell cycle arrest and apoptosis in the normal breast epithelial cell line MCF10A. BCE dysregulated polo-like kinase signaling and reduced

breast cancer cell proliferation-associated genes, including AURKA and KDM5B. These results suggest that blackcurrant anthocyanins may be useful as a component of breast cancer prevention.

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