Chemopreventive and Therapeutic Activity of Dietary Blueberry against Estrogen-Mediated Breast Cancer

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ABSTRACT: Berries are gaining increasing importance lately for their chemopreventive and therapeutic potential against several cancers. In earlier studies, a blueberry-supplemented diet has shown protection against 17β-estradiol (E2)-mediated mammary tumorigenesis. This study tested both preventive and therapeutic activities of diet supplemented with whole blueberry powder (50:50 blend of Tifblue and Rubel). Animals received 5% blueberry diet, either 2 weeks prior to or 12 weeks after E2 treatment in preventive and therapeutic groups, respectively. Both interventions delayed the tumor latency for palpable mammary tumors by 28 and 37 days, respectively. Tumor volume and multiplicity were also reduced significantly in both modes. The effect on mammary tumorigenesis was largely due to down-regulation of CYP 1A1 and ER-α gene expression and also favorable modulation of microRNA (miR-18a and miR-34c) levels. These data suggest that the blueberry blend tested is effective in inhibiting E2-mediated mammary tumorigenesis in both preventive and therapeutic modes.

KEYWORDS: blueberry, estrogen, breast cancer, chemoprevention, chemotherapy, ACI rats, miRNA modulation, blood chemistry, hematopoietic parameters

INTRODUCTION

Breast cancer is the most common cancer among American women and the second leading cause of cancer deaths. It is estimated that 232,340 women will be diagnosed with breast cancer and 39,620 will die in 2013.1 Accumulating data from epidemiological and experimental studies have shown that reproductive hormones, particularly E2, play a significant role in breast cancer etiology. Breast carcinogenesis is highly interlinked with the high levels of E2 and its metabolism to catechols mediated by cytochrome P450 enzymes.2 Other phase I and phase II enzymes involved in the metabolic activation and deactivation include estrone sulfatase, sulfotransferases, catechol-O-methyltransferase, and uridine-5'-diphosphate glucuronosyltransferase.3 The biological effects of E2 and its metabolites are mediated by two distinct E2 receptors (ER-α and ER-β) by binding with varying affinities.4

MicroRNAs (miRNAs) are small noncoding RNAs, and their expressions have been correlated with specific breast cancer pathological features such as E2 and progesterone receptor expression, proliferation index, and tumor stage.5 In an independent study, the expression of 667 miRNAs in 29 breast tumor and 21 adjacent normal tissues demonstrate 130 miRNAs to have significant differential expressions in breast tumors when compared to the normal adjacent tissues.6 miRNA expression is suppressed or stimulated by E2 and other E2 receptor ligands in human breast cancer cells, rat mammary gland, and other E2 responsive organs such as the endometrium and uterus.7

There is growing evidence from in vitro and in vivo studies that consumption of fruits and vegetables is associated with reduced risk of developing cancer.8 Berries are rich in anthocyanins, which are considered to be a good candidate for preventing the development of cancer by protecting cells from the damage caused by reactive oxygen species.9 There are over 500 anthocyanins characterized, but a great majority of them contain a core structure of six anthocyanidins, namely, cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin.10 Anthocyanins have mono-, di-, and tricyclic sugars attached to the anthocyanidin core structure and possess high antioxidant properties.11

Berries have received much attention lately due to their cancer chemopreventive potential. Blueberry (BB) is among the few fruits that contain five of the major anthocyanidins (cyanidin, delphinidin, malvidin, peonidin, and petunidin).12 Anthocyanins activate phase II enzymes and induce apoptosis as well as demonstrate antiproliferative, anti-inflammatory, and antiangiogenesis properties.13 In our recent study, an equimolar mixture of five major anthocyanidins was found to synergistically inhibit proliferation of two non-small-cell lung cancer cell lines both in cell culture and in vivo.14 In another study, we have demonstrated that dietary blueberry (Berkley) when provided in chemoprevention mode (5%, w/w) inhibited E2-mediated mammary tumorigenesis in ACI rats.15

In this study, we report on both chemopreventive and therapeutic potential of highbush blueberry powder (50:50 blend of Tifblue and Rubel). We also report the possible mechanism by which BB may inhibit mammary tumorigenesis.
MATERIALS AND METHODS

**Diet.** Freeze-dried highbush whole blueberry powder (50:50 blend of Tidblue and Rubel) was received from the U.S. Highbush Blueberry Council (Folsom, CA, USA). This blend contains 38 mg/g total phenolics and 21 mg/g total anthocyanins. AIN-93 M diet and AIN-93 M diet supplemented with 2.5 and 5% BB were purchased from pellet form by Harlan-Teklad (Madison, WI, USA) and stored at 4 °C in vacuum-sealed bags until use. BB diet was customized to have the same caloric value as AIN-93 M.

**Animal Study.** Animal experiments were executed in agreement with an approved protocol from the Institutional Animal Care and Use Committee at the University of Louisville. Female ACI rats (5–6 weeks old) were purchased from Harlan Sprague–Dawley, Inc. (Indianapolis, IN, USA). After a week of acclimation, animals were randomized into seven groups (Table 1). Groups 1, 4, and 7 were provided with AIN-93 M diet, and groups 2 and 5 were fed a diet supplemented with BB (2.5% w/w). Groups 3 and 6 received diet supplemented with 5% BB. After 2 weeks of experimental diet intervention, groups 4–7 were implanted subcutaneously with a 1.2 cm silastic implant containing 9 mg of 17β-estradiol (E2) as described. Group 7 received control AIN-93 M diet for 14 weeks and then was changed to BB diet (5% w/w) after the appearance of first palpable tumor in control group. As the BB diet in group 7 started after the appearance of tumors, this was considered as therapeutic intervention. Body weight and diet consumption were recorded weekly until euthanasia. Starting from 12 weeks of E2 treatment, animals were palpated weekly for tumor incidence and multiplicity. When the palpable tumors reached >90% in group 4, animals in all groups were euthanized by CO2 asphyxiation. Blood was collected by cardiac puncture to isolate serum and plasma. Lung, liver, brain, mammary tissue sections (5 μm mammary tissue sections (n = 21) from multiple previous studies were stained with hematoxylin and eosin and analyzed by a board-certified veterinary pathologist.

**Histopathology.** To assess the presence of microscopic tumors and pathological changes in the mammary glands of rats treated with E2 for 12 weeks, 5 μm mammary tissue sections (n = 21) from multiple previous studies were stained with hematoxylin and eosin and analyzed by a board-certified veterinary pathologist.

**Immunohistochemistry.** Tissue sections from five randomly selected animals from each group were stained for proliferating cell nuclear antigen (PCNA) using a PCNA staining kit (Life Technologies, Grand Island, NY, USA) following the manufacturer’s protocol. Five to six different sites in each slide were scored for deeply stained epithelial nuclei by two cytopathologists and represented as percent deeply stained cells.

**Plasma Prolactin.** Plasma prolactin levels were quantified using a Rat Prolactin ELISA Immuno-assay kit (Alpco, Salem, NH, USA) following the manufacturer’s instructions. Briefly, plasma samples (n = 5) from E2-treated groups were diluted 100- or 200-fold, and plasma from groups that did not receive E2 was diluted 2-fold prior to the analysis. The samples were added to a 96-well plate incubated with rat prolactin sample buffer followed by enzyme-labeled anti-rat prolactin antibody. Then samples were incubated with 3,3',5,5'-tetramethylbenzidine (TMB) and substrate solution (provided in the kit) followed by measurement of the optical density at 450 nm immediately after the addition of stop solution. About 25 μL of diluted plasma samples was measured against various concentrations of calibrator samples ranging from 5 to 80 ng/mL of prolactin levels.

**Western Blot Analysis.** Western blot analysis was done as described elsewhere. Briefly, whole-cell lysates were prepared from mammary tissues using radioimmunoprecipitation assay (RIPA) lysis buffer (Santa Cruz Biotechnology Inc., Dallas, TX, USA), and protein concentrations were measured by using the bicinchoninic acid (BCA) method. Proteins were separated on MES-SDS-Bis-Tris 4–12% gradient gel (Invitrogen, Carlsbad, CA, USA). The proteins were transferred to PVDF membranes and probed for cyclin D1, PCNA, estrogen receptor-alpha (ER-α), c-myc, and progesterone receptor (PR). Equal loading of the protein was confirmed by β-actin.

**Total RNA Isolation and qRT-PCR Analysis.** Total RNA from frozen mammary tissues was isolated using Trizol reagent (Invitrogen). RNA concentrations were measured by NanoDrop (N2000c, NanoDrop Corp., Wilmington, DE, USA). qRT-PCR was performed with 100 ng of RNA using a qScript One-Step SYBR Green qRT-PCR Kit (Quanta Biosciences, Gaithersburg, MD, USA) with 500 pmol of forward and reverse primers for each gene. The genes and primer sequences are as follows: CYP1A1 (forward, 5′ GAGCATGAGTGATGAT 3′; reverse, 5′ TGGAGACCCTCCGACATTCT 3′); ER-α (forward, 5′ GCCATGAGTTAACAAAGGCA 3′; reverse, 5′ GGATGAGACGAGTGAGCT 3′); 18S (forward, 5′ GGAGAATGTTGACGAAAATACAT 3′; reverse, 5′ TTGCCTCTCATTGATCCT 3′). Quantitative PCR was carried out on a 7500 Fast-Real Time PCR instrument (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s guideline. The gene expressions were calculated by relative quantification method (2^ΔΔCt) with control and normalizing to 18S RNA.

**Small RNA isolation/miRNA Analysis.** Small RNAs (<200 bp) were isolated from mammary tissues using a mirVana microRNA isolation kit (Invitrogen) following the manufacturer’s instruction. The concentration of the small RNA was quantified by Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). qPCR analysis of miR-18a, miR20a, miR25, and miR-34c was performed using a TaqMan miRNA Reverse Transcription Kit and TaqMan gene-specific miRNA assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Relative changes in miRNA expressions were quantified by comparison with the control and normalization to SS RNA.

**Toxicity Analysis.** Whole blood was collected at the time of euthanasia to analyze serum chemistry and hematological parameters, as described previously. Liver functions were analyzed by measuring the levels of aspartate transaminase, alanine aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, and amylase in serum. Kidney functions were analyzed by measuring blood urea nitrogen, creatinine, and various electrolytes in serum. Various hematological parameters such as white blood cells (WBCs), red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count were analyzed from whole blood. Five random samples from each group were analyzed.

**Statistical Analysis.** All statistical comparisons between controls and E2-treated groups were made using an unpaired one-tailed Student’s t test. Tumor latency was analyzed using a nonparametric log-rank test. Diet consumption was analyzed using an unpaired two-tailed Student’s t test, and body weight was analyzed by two-way ANOVA. All statistical analyses were done in GraphPad prism software, version 4.3 (San Diego, CA, USA). A p value of ≤0.05 was considered significant.

**RESULTS**

**Food Consumption and Body Weight.** There was no significant difference in diet consumption among groups receiving either control diet or AIN-93 M diet supplemented with 2.5 and 5% BB, in the absence of E2 treatment. The diet consumption, however, increased in E2-treated animals fed control diet or BB-supplemented diets (Figure 1A). The

Table 1. Experimental Design

| group | treatment | no. of animals |
|-------|-----------|----------------|
| 1     | untreated | 8              |
| 2     | 2.5% BB diet | 5                |
| 3     | 5% BB diet | 5              |
| 4     | E2 + control (AIN-93 M diet) | 24               |
| 5     | E2 + 2.5% BB diet | 20             |
| 6     | E2 + 5% BB diet (preventive) | 20             |
| 7     | E2 + 5% BB diet (therapeutic) | 20            |
increase was significant in animals receiving the 5% BB diet in both preventive and therapeutic modes; the increase was insignificant in the 2.5% BB diet group. At the end of the study (25 weeks), one-way ANOVA analysis showed no significant difference in body weights among the control and E2-treated animals (Figure 1B).

**Tumor Incidence, Multiplicity, and Volume.** BB diet was highly effective in favorably modulating the various tumor indices (latency, incidence, multiplicity, and burden). BB diet dose dependently delayed the tumor latency. The groups receiving 5% BB diet in both the chemopreventive and therapeutic modes had a significant delay with the first tumor appearance in the control group by 28 and 37 days, respectively, as compared to the age-matched control animals (Figure 2A). The group that received 2.5% BB diet also had delayed tumor latency but only slightly, about 7 days. At the end of the study, the control group developed palpable mammary tumors in 96% animals, whereas animals provided with the 5% BB diet in chemopreventive and therapeutic groups had palpable tumors only in 60% (p = 0.0109) and 55% (p = 0.0021) of animals, respectively; the 2.5% BB diet also showed some (11%) but insignificant reduction in tumor incidence compared to the control group. Tumor volume and multiplicity were measured after the animals had been euthanized. Animals receiving control diet had an average tumor volume of 979 ± 218 mm$^3$, whereas animals on the 5% BB diet had significant reductions, 512 ± 130 mm$^3$ (p = 0.0398) and 452 ± 144 mm$^3$ (p = 0.0106), for chemopreventive and therapeutic mode, respectively (Figure 2B). Animals on the 2.5% BB diet showed a modest but insignificant
Table 2. Comparison of Body Weight and Other Organ Weights between ACI Rats Fed Control Diet or Diet Supplemented with Blueberry

| group | body weight (g) | liver (g) | mammary (g) | pituitary (mg) |
|-------|----------------|-----------|-------------|---------------|
| untreated (n = 8) | 189.4 ± 6.8 | 4.9 ± 0.4 | 4.6 ± 0.9 | 11.2 ± 1.3 |
| 2.5% BB diet (n = 5) | 192.6 ± 15.9 | 5.2 ± 1.0 | 4.4 ± 0.9 | 12.2 ± 1.0 |
| 5% BB diet (n = 5) | 185.0 ± 6.6 | 4.8 ± 0.4 | 4.6 ± 0.4 | 9.8 ± 1.4 |
| E2 + control AIN 93 M diet (n = 24) | 189.0 ± 14.2 | 6.3 ± 0.4 | 6.4 ± 1.5 | 42.3 ± 10.7 |
| p value | | | 0.0001 | |
| E2 + 2.5% BB diet (n = 18) | 192.3 ± 12.0 | 6.5 ± 0.6 | 5.7 ± 1.5 | 34.4 ± 8.0 |
| p value | | | 0.007 | |
| E2 + 5% BB diet (preventive) (n = 18) | 191.2 ± 17.5 | 6.7 ± 0.8 | 5.6 ± 2.0 | 33.9 ± 5.6 |
| p value | | | 0.0486 | 0.0031 |
| E2 + 5% BB diet (therapeutic) (n = 18) | 192.4 ± 12.0 | 6.5 ± 0.5 | 5.9 ± 1.2 | 34.4 ± 5.3 |
| p value | | | 0.0031 | |

“Body weight and organ weight values are expressed as mean ± SD. "Values that are significantly higher than untreated control. "Values that are significantly higher than E2-treated animals. Average body weight of animals is measured at the time of euthanasia.

Figure 3. Presence of epithelial dysplasia and ductular papillomas from mammary tissue of rats treated with E2 implant for 12 weeks. (A) Image of mammary section at 40× magnification showing long papillary projections from ductular walls (thin arrows) and ducts lined with hyperplastic epithelium with two or more layers (bold arrows). Inset shows 400× magnification of papillary projections. (B) Mammary section at 600× magnification with nuclei showing karyomegaly, which are hyperchromatic containing one to three distinct nucleoli (thin arrows).

(0.1435) reduction with an average tumor volume of 698 ± 145 mm³. Tumor multiplicity in control animals was 4.17 ± 0.48 per animal, and it was reduced to 2.17 ± 0.52 (p = 0.0039) and 1.75 ± 0.45 (p = 0.0004) by the 5% BB diet given in chemopreventive and therapeutic mode, respectively (Figure 2C); the 2.5% BB diet also showed a significant reduction in tumor multiplicity, 2.75 ± 0.45 (p = 0.0199).

Liver, Mammary, and Pituitary Weights. At the end of the study, the liver, mammary, and pituitary tissue weights were not significantly different among the groups without E2 treatment. However, the differences were significant with E2 treatment. Mammary tissue weight with 5% BB diet in chemopreventive mode was modestly offset (p = 0.0486) from E2-treated control. There was a 4–5-fold increase in the pituitary weight in E2-treated animals; however, this was significantly offset with the 5% BB diet in preventive (33.9 ± 5.6 mg vs 42.2 ± 10.7 mg; p = 0.0031) and therapeutic (34.4 ± 5.3 mg; p = 0.0031) modes (Table 2). Pituitary weights in animals receiving the 2.5% BB diet also showed significant decrease (34.4 ± 8.0 mg; p = 0.0070).

Histopathology. In all animals that were treated with E2 for 12 weeks (n = 21), there was expansion of mammary tissue with proliferation of terminal buds that differentiated into acini. The acinar epithelial cells were frequently vacuolated, and the acini contained amphophilic inspissated secretory material filling the lumina (Figure 3A). Concomitantly, there was proliferation of ductules and ducts with variable grades of epithelial dysplasia (n = 13). The ductular epithelial cells were cuboidal with distinct cell borders and moderate amounts of eosinophilic cytoplasm. Often (n = 4), the nuclei showed karyomegaly and were hyperchromatic, containing one to three distinct nucleoli (Figure 3B). Mitotic figures were not present in the sections examined. In >50% of cases (n = 11), the ducts were ectatic and lined by hyperplastic epithelium that formed two or more layers (Figure 3A). Ductular adenomas (n = 3) were characterized by the development of a 100–250 μm long papillary projection from the ductular walls that narrowed the lumina (Figure 3A, inset). The neoplastic cells contained scant cytoplasm and round nuclei with one to three distinct nucleoli. In all cases, desquamated epithelial cells were present within the ductular lumina. Prominent periductular fibrosis was a feature of all adenomas. Multifocal perivascular mast cell infiltration correlated with the severity of the lesion in all cases. In addition, the ductular epithelial dysplasia correlated with the size of expansion of the terminal bud.

Cell Proliferation Index. Cell proliferation was analyzed by staining tumor and adjacent mammary tissue for PCNA protein markers using antibody-based assay. After 25 weeks, there was no significant difference in cell proliferation among animals receiving either control or 2.5 and 5% BB diets. However, the E2 treatment increased the cell proliferation to nearly 6-fold (p = 0.0095) (Figure 4). This increase in the cell proliferation was
significantly offset by 34 and 35% with 5% BB diet administered in chemopreventive and therapeutic mode, respectively (Figure 4); the effect was insignificant with the 2.5% BB diet.

Plasma Prolactin. No significant difference was observed in the levels of plasma prolactin between untreated groups and BB diet groups. On the other hand, there was a substantial increase ($p = 0.0035$) in prolactin levels with E2 treatment (6973 ± 2443 ng/mL) compared to untreated control (11.8 ± 1.8 ng/mL). After 25 weeks, animals provided with 5% BB diet in chemopreventive (2205 ± 438 ng/mL; $p = 0.05$) and therapeutic mode (1950 ± 285 ng/mL; $p = 0.01$) demonstrated significant reduction in the elevated levels of plasma prolactin (Figure 5). A modest but insignificant reduction in prolactin levels (4193 ± 1509 ng/mL) was also observed with the 2.5% BB diet.

Modulation of Estrogen-Specific Genes. There was a pronounced effect of E2 on the expressions of cytochrome CYP1A1 and ER-α at the mRNA levels. All groups were compared against the untreated control, and the fold change was expressed as mean ± standard error. E2 treatment up-regulated the CYP1A1 mRNA levels by 60-fold. The 5% BB intervention provided in chemopreventive and chemotherapeutic modes both significantly offset the E2-associated increase in CYP1A1 to 28.3 ± 6-fold ($p = 0.0011$) and 27.6 ± 10.3-fold ($p = 0.0102$), respectively. Similarly, the mRNA expression level of ER-α in E2-treated animals was up-regulated by 19.7 ± 2-fold compared to untreated control, and this increase was also significantly offset by the 5% BB diet, chemopreventive mode (14.2 ± 2-fold; $p = 0.0201$) and therapeutic mode (11.1 ± 4-fold; $p = 0.05$) (Figure 6A). Western blot analysis showed that E2 treatment up-regulated both cyclin D1 and PCNA. Intervention of 5% BB diet in both preventive and therapeutic modes down-regulated cyclin D1 and PCNA at protein levels.

However, ER-α protein level was not modulated with either E2 treatment or administration of BB diet.

Modulation of Estradiol-Specific miRNAs. We analyzed four E2-specific miRNAs (miR-18a, -20a, -25, and -34c) on the basis of our previous study in this animal model to determine the effect of BB treatment on their modulation. All four miRNAs were significantly up-regulated under the influence of E2 intervention in agreement with our previous findings. miR-18a and miR-34c were up-regulated about 13- and 11-fold, respectively, compared to untreated control.
miR-18a and miR-34c was reduced to 6.4-fold \((p = 0.0013)\) and 4.9-fold \((p = 0.0033)\), respectively, with the 5% BB diet in therapeutic mode (Figure 7). Berry diet given in chemo-

**Table 3. Serum and Hematological Parameters for Assessment of Toxicity and Tolerance of Blueberry Diet**

| Parameter                  | untreated       | 5% BB diet      | E2 + control AIN-93 M diet | E2 + 5% BB diet |
|----------------------------|-----------------|-----------------|----------------------------|-----------------|
|                            |                 |                 | preventive                 | therapeutic     |
| serum enzymes              |                 |                 |                            |                 |
| AST (SGOT)                 | 342 ± 130       | 276 ± 80        | 236 ± 31                   | 217 ± 51        |
| ALT (SGPT)                 | 56 ± 9          | 38 ± 5          | 45 ± 10                    | 49 ± 7          |
| alk phosphatase            | 31 ± 9          | 41 ± 8          | 33 ± 17                    | 32 ± 9          |
| GGT                        | 8 ± 2           | 3 ± 0           | 5 ± 2                      | 4 ± 1           |
| amylase                    | 552 ± 91        | 585 ± 40        | 548 ± 72                   | 585 ± 130       |
| lipase                     | 25 ± 0          | 30 ± 25         | 25 ± 0                     | 26 ± 1          |
| plasma proteins            |                 |                 |                            |                 |
| albumin (g/dL)             | 4 ± 0.1         | 4 ± 0.3         | 4 ± 0.1                    | 4 ± 0.4         |
| globulin (g/dL)            | 3 ± 0.5         | 3 ± 0.3         | 4 ± 0.3                    | 4 ± 0.2         |
| glucose (mg/dL)            | 122 ± 36        | 139 ± 46        | 90 ± 30                    | 118 ± 33        |
| cholesterol (mg/dL)        | 118 ± 14        | 98 ± 10         | 131 ± 16                   | 124 ± 20        |
| triglycerides (mg/dL)      | 103 ± 27        | 108 ± 20        | 91 ± 23                    | 99 ± 28         |
| kidney function            |                 |                 |                            |                 |
| BUN (mg/dL)                | 24 ± 10         | 21 ± 3          | 21 ± 4                     | 20 ± 4          |
| creatinine (mg/dL)         | 0.4 ± 0.1       | 0.5 ± 0         | 0.44 ± 0.1                 | 0.44 ± 0.1      |
| phosphorus (mg/dL)         | 16 ± 6          | 19 ± 2          | 13 ± 4                     | 13 ± 2          |
| calcium (mg/dL)            | 11 ± 1          | 12 ± 1          | 12 ± 3                     | 12 ± 0.3        |
| magnesium (mequiv/dL)      | 4 ± 0           | 4 ± 0.3         | 3 ± 0.3                    | 3 ± 0.3         |
| sodium (mequiv/dL)         | 146 ± 5         | 147 ± 4         | 144 ± 1                    | 141 ± 2         |
| potassium (mequiv/dL)      | 8 ± 4           | 9 ± 1           | 8 ± 0.9                    | 7 ± 0.3         |
| chloride (mequiv/dL)       | 81 ± 42         | 100 ± 2         | 98 ± 3                     | 97 ± 2          |
| hematological parameters   |                 |                 |                            |                 |
| WBC (10^3/μL)              | 5 ± 2           | 4 ± 0.3         | 5 ± 2                      | 6 ± 3           |
| RBC (10^6/μL)              | 8 ± 0.1         | 8 ± 0.5         | 6 ± 1                      | 6 ± 2           |
| HGB (g/dL)                 | 13 ± 0.3        | 13 ± 1          | 9 ± 4                      | 10 ± 2          |
| HCT (%)                    | 44 ± 3          | 46 ± 2          | 35 ± 3                     | 33 ± 7          |
| MCV (fL)                   | 55 ± 3          | 55 ± 3          | 58 ± 4                     | 55 ± 3          |
| MCH (pg)                   | 17 ± 0.3        | 16 ± 0.3        | 17 ± 1                     | 16 ± 1          |
| MCHC (g/L)                 | 30 ± 2          | 29 ± 2          | 29 ± 1                     | 30 ± 1          |
| hematocrit (%)             |                 |                 |                            | 20 ± 8          |
| platelet count (10^3/μL)   | 854 ± 100       | 807 ± 334       | 872 ± 140                  | 648 ± 216       |
| neutrophils                | 634 ± 287       | 684 ± 203       | 2106 ± 1608                | 2229 ± 1394     |
| lymphocytes                | 4222 ± 1882     | 3475 ± 666      | 2347 ± 675                 | 3102 ± 1628     |

*ND, not determined.*
neutrophils and lymphocytes measured in whole blood showed no significant difference among all groups (Table 3).

**DISCUSSION**

Cancer chemopreventive agents are either natural products or their synthetic analogues, which inhibit the transformation of normal cells to premalignant cells or the progression of premalignant cells to malignant cells. It is believed that chemopreventive agents can inhibit oncogenic pathways, inhibit growth, and induce apoptosis in cancer cells. Conventional chemopreventive agents are administered long-term and believed to block or delay the progression of transformed cells by modulating cell proliferation or differentiation. They also prevent activation of carcinogen, trap carcinogen before reaching the active site, and enhance detoxification systems. On the other hand, standard therapeutic drugs are toxic to both cancer and normal cells, which results in therapeutic activity and side effects to normal cells. Cytotoxicity to normal cells may lead to secondary tumors.

This study was conducted to test both the chemopreventive and therapeutic potential of Tifblue and Rubel blueberry blend against E2-mediated mammary tumorigenesis. We had previously reported on the chemopreventive effect of Berley blueberry on mammary tumorigenesis. This is the first study of its kind in which the therapeutic potential of blueberry has been shown. We also confirmed the microscopic tumors and precancerous conditions by histological evaluation of mammary gland sections from rats treated and necropsied at 12 weeks of E2 treatment. Variable ductular epithelial changes including dysplasia, hyperplasia, and adenoma formation were found, suggesting various stages of carcinogenesis. In addition, periductular fibroplasia as well as mast cell infiltration positively correlated with degree of dysplasia.

Similar to our earlier studies, this Tifblue–Rubel blueberry blend at 2.5 and 5% given in the chemopreventive mode was highly effective in increasing the tumor latency by 7 and 28 days, respectively. The administration of 2.5% BB diet showed significant reduction in tumor multiplicity and pituitary weight. In our earlier studies, 2.5% BB diet made from the mixture of three blueberries was not effective against E2 treatment; this could be because the E2 implant size was 3 cm long containing 27 mg of E2. In another study with a similar 9 mg E2 implant, a 2.5% diet showed protective effects that were in the same vicinity as reported by us previously. In both studies, the 2.5% BB diet had only modest effect and did not significantly reduce the tumor volume, tumor latency, and molecular markers investigated. The higher dose of BB (5%) administered in chemopreventive mode was highly protective in reducing tumor volume and tumor multiplicity and increased tumor latency to 28 days, in agreement with our previous findings. However, the protective effect of blueberry was slackened after 20 weeks of E2 treatment, likely due to the exponential growth rate of the cells to reach the malignant stage. For the first time BB diet was administered after 12 weeks of E2 treatment to determine the therapeutic effect against precancerous and microscopic adenomas. When compared with animals that received 5% BB diet in chemopreventive mode, the therapeutic group showed a significantly enhanced effect by reducing tumor volume and multiplicity and increasing the tumor latency to 37 days, that is, 9 days more than the preventive groups. Even when the BB diet was initiated after 14 weeks of E2 treatment, the therapeutic group elicited protective effects. The therapeutic potential of BB anthocyanidins has been shown by others against human prostate cancer and by us against lung cancer xenograft. However, there is no study for direct comparison in E2-induced mammary tumorigenesis. Interestingly, the present data also suggest that BB diet can be started in the postinitiation stage of tumorigenesis and still elicit a response similar to that of chemopreventive mode.

Bioavailability of anthocyanins in vivo is crucial for their biological effects. Anthocyanins are absorbed intact in the glycosidic form. They are rapidly absorbed from the stomach and jejunum. Yet >50% of the ingested anthocyanins are found in the cecum, implying poor absorption. Anthocyanin structure exists as a flavilium cation in acidic conditions, which is stable, but converts to unstable quinoidal or carbilin structure at neutral or alkaline conditions. However, it has also been shown that anthocyanins are stabilized by flavanols and ascobic acid present in the food matrix. This may explain the higher bioavailability of anthocyanins when given as a whole fruit powder relative to individual anthocyanins, indicating strong synergism occurs in absorption between coexisting molecules in fruit. Furthermore, in a study with healthy subjects with ileostomy, nearly 40% of anthocyanins were recovered in the ileal fluid, indicating that despite the difference in pH, the anthocyanins were stable and large amounts will pass from the small intestine to the large intestine. Yet, all of the bioavailability studies were done with bolus doses of pure anthocyanins or fruit powder/extracts. Anthocyanins were not detected in the plasma of animals fed a diet mixed with sources rich in anthocyanins or the pure compound.

However, lack of detection of anthocyanins in the plasma does not indicate lack of tissue bioavailability. When anthocyanins were delivered intravenously, a biphasic decay of plasma levels was observed, indicating tissue distribution. In a parallel publication in this issue, we have discussed in detail the bioavailability of anthocyanins and its levels in lung tissue. We have demonstrated the presence of all five anthocyanidins, delphinidin, cyanidin, peonidin, petunidin, and malvidin, in lung tissues. Thus, anthocyanins are bioavailable and bioaccumulate in non-gastrointestinal tissues.

In agreement with our previous studies, the effect of E2 was reflected in the body, liver, mammary, and pituitary weights of the animals. The transitory increase in body weight and organ weight is majorly due to the E2 and may not be due to the diet consumption. Moreover, control AIN-93 M diet and diets supplemented with 2.5 and 5% BB were isocaloric. Although there was no effect of BB diet on body weight gain and organ weights, it was found to significantly offset E2-associated elevation in the weight of pituitary tumors. The pituitary weight was affected due to the increase in plasma prolactin levels induced by E2, resulting in pituitary prolactinomas. However, the E2-mediated mammary tumor model was well studied and standardized to reduce the side effect to the pituitary gland. Earlier studies have shown that prolonged supply of E2 increases the production of prolactin in E2-sensitive rat strains. High circulatory levels of prolactin in serum are one of the major reasons for animals to develop E2-induced mammary tumors. Berry diet at 2.5 and 5% significantly offset the prolactin levels elevated by E2.

E2 also induces the production of phase I enzyme, CYP1A1. It catalyzes the conversion of E2 into its metabolites (2E2 and 4E2), which further undergo redox cycle leading to the formation of reactive oxygen species. In this study, we...
showed that BB diet significantly reduced the expression of CYP1A1, suggesting its protective role against mammary carcinogenesis. Studies have shown that cyclin D1 and E2 receptors have important roles in regulating proliferation of breast epithelial cells.\(^{1,42}\) It is well established that E\(_2\) via ER-\(\alpha\) elicits rapid signals driving cancer cells to proliferation. This biological effect of E\(_2\)-mediated by ER-\(\alpha\) was reduced significantly in 5% BB diet provided in therapeutic mode. Regulation of cyclin D1 gene expression has been associated with changes in the proliferation rate of breast cancer cells.\(^{43}\) Induction of cyclin D1 expression occurs rapidly and is a critical feature of the mitogenic action of E\(_2\). The expression of cyclin D1 was down-regulated by 2–2.6-fold with 5% BB diet in chemopreventive and therapeutic modes, respectively. Investigation of PCNA in nuclear proteins revealed that dietary BB reduced the expression levels significantly. PCNA was shown to interact with ER-\(\alpha\) both in the absence and in the presence of DNA, to enhance the interaction of ER-\(\alpha\) with ERE-containing DNA, and associate with endogenous E2-responsve genes.\(^{44}\) These findings clearly indicate that BB diet could modulate major E\(_2\)-associated proliferation markers that lead to protective effects against mammary tumorigenesis in animals treated with E\(_2\).

Another interesting finding in this study is from the analysis of miRNAs related to the ER-\(\alpha\) family. Aberrant miRNA expression is implicated in E\(_2\)-related breast, uterine, and ovarian cancer development and progression.\(^{45}\) Hormones play a crucial role in regulating miRNAs by both genomic (transcriptional) and nongenomic mechanisms.\(^{45}\) Lorio et al. identified miRNAs having expression correlated with specific breast cancer biopathologic features, such as E\(_2\) and progesterone receptor expression, tumor stage, vascular invasion, or proliferation index.\(^{46}\) Targeting specific miRNAs for inhibiting specific stages of a cancer\(^{40}\) is being explored. Several miRNAs that regulate ER are involved in synchronized feedback mechanisms as a component of ER activation. Many of these ER-regulating miRNAs therefore are either E\(_2\)-inducible (miR-18a and let-7) or subject to E\(_2\)-mediated repression (miR-206, miR-221/222, and miR-145).\(^{47}\) This laboratory has recently identified miRNAs that modulated under the influence of E\(_2\) in this animal model and report aberrant changes in miRNA expression profile as early as 3 weeks of E\(_2\) treatment.\(^{48}\) We chose four miRNAs, miR-18a, -20a, -25, and -34c, on the basis of our previous study where these miRNAs were up-regulated with E\(_2\) to understand the influence of BB diet on their modulation. Increases in miR-18a and miR-20a mature forms were observed after E\(_2\) stimulation.\(^{18}\) In this study, BB diet administered in therapeutic mode significantly reduced miR-18a and miR-34c, whereas chemopreventive mode showed modest but significant reduction. These findings provide initial insights of BB diet in decreasing the E\(_2\)-induced miRNA levels. However, the BB diet failed to modulate miR-20a and -25 expression levels, which remain the subject of further investigation.

In the chemopreventive mode, the rats are fed BB diet as young as 6–7 weeks old. The relative enhanced effect observed with the therapeutic approach could be due to the adaptive response of the rats to the BB phytochemicals in the chemopreventive mode. For example, the CYP1A1 mRNA level in mammary tissue was highest at 3 weeks after E\(_2\) implant and gradually decreased with time.\(^{15,23}\) The effect of the BB phytochemicals were more pronounced in the inhibition of conversion of normal cells to precancerous or precancerous frank tumors, but were not as effective once the tumors reached the exponential growth rate. Thus, by the time the mammary cells started exhibiting precancerous conditions, the efficacy was better when BB phytochemicals were introduced into the system than when they were already in the circulation. Nevertheless, both approaches were effective in delaying the onset and growth of palpable tumors.

In summary, this is the first demonstration indicating that BB diet is highly protective even in preinitiated E\(_2\)-mediated mammary tumors in ACI rats through its effects by modulating cell proliferation and molecular targets. Thus, the consumption of BB can be used as an effective strategy for the treatment of E\(_2\)-associated breast cancer and possible prevention of its relapse and metastasis. Our study demonstrates the chemopreventive and therapeutic potential of blueberry and advances our understanding of the working mechanisms in search of potential future drugs.

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**Notes**

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