In utero and lactational exposure to blueberry via maternal diet promotes mammary epithelial differentiation in prepubescent female rats

Xianli Wu, Omar Rahal, Jie Kang, S. Renee Till, Ronald L. Prior, Rosalia C.M. Simmen

Abstract

Early developmental events influence the fine tuning of later susceptibility to adult diseases. Diet is a determinant of breast cancer risk, and our previous studies showed that diet-mediated changes in transcriptional programs promote early mammary gland differentiation. Although consumption of fruits is considered to elicit multiple health benefits, little is known on whether associated bioactive components modify the early differentiation program in developing mammary glands. Here, we evaluated the hypothesis that early exposure (in utero and lactational) to blueberry through maternal diet enhances mammary epithelial differentiation in female offspring. Pregnant Sprague-Dawley rats beginning at gestation day 4 were fed American Institute of Nutrition–based diets containing casein and whole blueberry powders added to casein at 2.5%, 5.0%, and 10% weight/weight. Female pups at weaning were evaluated for growth and mammary tissue parameters. Blueberry at 5% dose increased body and adipose fat weights, relative to the other diets. Mammary branch density and terminal end bud size were highest for the 5% blueberry group, whereas terminal end bud numbers were not affected by all diets. Mammary ductal epithelial cells of the 5% blueberry group had lower nuclear phosphorylated histone 3 and higher nuclear tumor suppressor phosphatase and tensin homolog deleted in chromosome 10 (PTEN) levels than the casein group. Although sera of both diet groups had similar antioxidant capacity, 5% blueberry sera elicited higher nuclear PTEN accumulation in human MCF-10A mammary epithelial cells. Our studies identify developing mammary glands as early targets of blueberry-associated bioactive components, possibly through systemic effects on epithelial PTEN signaling.

Keywords: Blueberry; Mammary gland; PTEN; Puberty; Rat

Abbreviations: BB, blueberry; CAS, casein; PND, postnatal day; PTEN, phosphatase and tensin homolog deleted in chromosome 10; TEB, terminal end bud.

1. Introduction

The concept that early life experiences are linked to the development of adult chronic diseases has its roots in the seminal findings of Prof David Barker and colleagues on the association between low birth weight and increased risk for adult type 2 diabetes mellitus [1]. Although emerging evidence suggests that other diseases such as breast cancer are similarly subject to considerable influence by early developmental events [2-5], the mechanisms underlying “developmental plasticity” remain poorly understood. In the case of breast cancer, a leading cause of malignancy among...
women [6], understanding the biological basis of this phenomenon is even more challenging, given the many contributing elements to its multiple histopathologies [7-10]. Among all cancer types, breast cancer is considered to have the greatest chance of detection and effective treatment; however, primary prevention of this disease by adopting healthier lifestyles has gained widespread support. In principle, the protective effects of bioactive components present in foods could result from promotion of the differentiation of the highly proliferative terminal end buds (TEBs) of the mammary gland, whose numbers are maximal at prepuberty and increasingly decrease and disappear thereafter in adult tissues [11]. Terminal end buds are presumed to contain the stem cell population [12] from which epithelial tumors originate, when these cells undergo overexpansion [13-15]. Nevertheless, other mammary compartments constitute potential targets of dietary factors as well [16].

Consumption of fruits as part of the regular diet is considered good nutritional practice, with health advocates pushing for 5 to 9 servings of fruits and vegetables a day. Although solid scientific evidence for a linkage between breast health and high intake of fruit is lacking, one case-controlled study [17] and one prospective study [18] in women provided data in support of an association of high fruit (and vegetable) intake with reduction in breast cancer risk. Among fruits, berries are valued for their oncoprotective activities, based largely on animal and human tumor cell–based studies [19-23]. Although these antitumor activities can be partly attributed to the high anthocyanin and polyphenol content of berries [24] that display high oxygen radical absorbance capacity to reduce cellular DNA damage, there are likely additional yet unknown underlying mechanisms.

The dual (protein/lipid) phosphatase, phosphatase and tensin homolog deleted in chromosome 10 (PTEN) is the second most frequently mutated tumor suppressor gene in human cancers [25-27]. PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate to negatively regulate the PI3-kinase/Akt pathway [28]. Diminished PTEN function leads to increased proliferation and reduced apoptosis, both hallmarks of mammary tumorigenesis, and primary ductal adenocarcinomas of the breast display loss of PTEN expression [26,27]. Recent findings suggest a nuclear function for PTEN in tumor suppression and chromosomal stability [29]. The reported positive association between PTEN dosage and tumor phenotype in humans suggests that reactivation of PTEN expression may have important clinical utility [30].

The rat mammary gland constitutes a highly relevant model for studying the biological actions of dietary compounds, given its many similarities to human breast in structure, function, and development [31,32]. Ductal and lobuloalveolar development in the rat mammary gland occurs extensively at peripuberty, similar to human females. In the present study, we evaluated the hypothesis that early exposure (in utero through lactation) to blueberry via maternal diet enhances mammary epithelial differentiation in female rat offspring. To address this, we examined the effects of early exposure to dietary intake of whole blueberry (BB) by pregnant and lactating dams on mammary architecture and differentiation marker PTEN expression in prepubertal female rats at weaning. We also determined whether systematic changes due to early exposure to dietary BB underlie enhanced mammary epithelial differentiation, involving nuclear PTEN accumulation. Our findings provide the first evidence of the early influence of maternal BB diet on mammary gland development of offspring and suggest that breast health in women may be similarly enhanced by increased fruit intake by pregnant and lactating mothers.

2. Methods and materials

2.1. Rats, diets, and tissue collection

Animal care and handling followed protocols approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences. Time impregnated Sprague-Dawley rats (Charles River Laboratories, Inc, Wilmington, Mass) were maintained in the animal facility of the Arkansas Children’s Nutrition Center in a temperature- and humidity-controlled room. Pregnant females beginning at gestation day 4 were randomly assigned to the American Institute of Nutrition-93G–based pelleted diet containing casein (CON) as the major protein source to which was added freeze-dried powders of blueberry (BB) at 2.5%, 5%, and 10% by weight of feed. Freeze-dried BB powders were provided by FutureCeuticals Inc (Momence, Ill). Diet with added BB was formulated to be isoenergetic and isonitrogenous by adjusting the amounts of casein, corn starch, and cellulose fiber (Table 1). The chemical composition of the whole BB powder is presented in Table 2. All rats were provided food and water ad libitum. The amount of food absorbed capacity to reduce cellular DNA damage, there are likely additional yet unknown underlying mechanisms.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Ingredient composition of the diets</th>
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<tbody>
<tr>
<td>Component (g/Kg)</td>
<td>CON</td>
</tr>
<tr>
<td>Casein</td>
<td>200.0</td>
</tr>
<tr>
<td>l-Cystine</td>
<td>3.0</td>
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<tr>
<td>Corn starch</td>
<td>397.5</td>
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<tr>
<td>Maltodextrin</td>
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<tr>
<td>Sucrose</td>
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<tr>
<td>Corn oil</td>
<td>70.0</td>
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<tr>
<td>Cellulose</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mix, AIN-93G-MX (TD 94046)</td>
<td>10.0</td>
</tr>
<tr>
<td>Vitamin mix, AIN-93G-VX (TD 94047)</td>
<td>0.014</td>
</tr>
<tr>
<td>Blueberries</td>
<td>2.5</td>
</tr>
</tbody>
</table>

AIN indicates American Institute of Nutrition.
consumed among pregnant dams, and litter size for all dams were comparable for all dietary groups (data not shown). Weight gain among pregnant dams (from gestation days 3 to 17) across all diets did not significantly differ (Fig. 1). Pregnant rats were maintained on the same diets throughout gestation and lactation. At delivery (postnatal day [PND] 0), pups from dams of the same dietary group were pooled, and 10 pups (5 per sex) were randomly assigned to each dam for suckling. Female pups were euthanized at weaning (PND21) in a carbon dioxide inhalation chamber. Serum samples prepared from individual rats were stored at −20°C for further analyses (below). Body, uterine, and abdominal fat weights and vaginal opening (measure of sexual maturity) were measured for females of all dietary groups. The inguinal mammary gland pair (gland 4) was removed for \( n = 8 \) rats per diet group. The left gland was processed for whole mounts, whereas the right gland was fixed for paraffin embedding and subsequent histologic analyses [16,33].

### 2.2. Mammary whole mounts, adipocyte size, and immunohistochemistry

Mammary gland whole mounts were prepared after previously described protocols [34]. Histologic analyses of glands were performed using \( n = 6 \) to 8 rats per dietary group. The numbers of TEBs per unit area (27 mm\(^2\)) were determined in 4 to 5 areas per slide. The epithelial area of TEBs from paraffin-embedded glands was determined by outlining individual TEBs using the imaging software freeware tool of the Olympus BX50 microscope, and subtracting the areas of luminal and other vacuolated spaces, as previously published [35]. Branching density for each gland was quantified by counting the number of branchpoints within a box of fixed dimensions, following previously described protocols [35]. To determine adipocyte cell size, tissue sections from paraffin-embedded glands were stained with hematoxylin and eosin [16]. Adipocyte areas were measured in 2 to 3 randomly selected fields per slide (200-300 cells per field) using Axiovision software (Carl Zeiss AG, Oberkochen, Germany) [16]. Immunohistochemical staining of phosphorylated histone H3 were evaluated using anti-phospho-histone H3 (Ser 10) antibody (no. 9710; Cell Signaling, Danvers, Mass, USA), following the manufacturer’s protocols. Immunoreactive PTEN expression in nuclear and cytoplasmic compartments of mammary ductal epithelium was evaluated using anti-PTEN antibody (A2B1, Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), as previously described [36]. The numbers of intensely staining nuclei in ductal epithelium and TEBs were counted from a total of 300 to 400 cells per tissue section per rat.

### 2.3. Cell culture and PTEN immunofluorescence

The nontumorigenic human mammary epithelial cell line MCF10-A was obtained from American Type Culture
Cells were then incubated with biotinylated secondary antibody (A2B1; 1:200 dilution) overnight at 4°C. PTEN antibody (PTEN; Vector Laboratories, Burlingame, Calif), and incubated with anti-PTEN antibody at 5-fold stoichiometric excess for 2 hours at room temperature, and the mixture was incubated with media change and processed 24 hours after the last treatment. To demonstrate specificity of PTEN immunoreactivity, a PTEN blocking peptide (sc-7974P, Santa Cruz) was incubated with PTEN antibody in place of the antibody alone. Cells were treated twice (at 0 and 24 hours) with phenol red–free medium containing sera (to 1% final concentration) pooled from PND21 pups (n = 4 per diet group) exposed to control (0% BB) and 5% BB through maternal diets. Cells were treated twice (at 0 and 24 hours) with media change and processed 24 hours after the last treatment. To demonstrate specificity of PTEN immunoreactivity, a PTEN blocking peptide (sc-7974P, Santa Cruz) was incubated with PTEN antibody at 5-fold stoichiometric excess for 2 hours at room temperature, and the mixture was applied to tissue sections in place of the antibody alone. Cells were fixed, permeabilized in methanol, blocked in 1% horse serum blocking solution (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, Calif), and incubated with anti-PTEN antibody (A2B1; 1:200 dilution) overnight at 4°C. Cells were then incubated with biotinylated secondary antibody (Vectastain Elite ABC kit), followed by rhodamine (tetramethylrhodamineisothiocyanate)-conjugated strepavidin (1:250 dilution; Jackson ImmunoResearch Laboratories, West Grove, Pa). Subsequent processing of slides and analyses of fluorescent images were as previously described [38]. The percentage of nuclear PTEN-staining cells relative to the total number of cells counted (~350 cells/slide) were quantified in 2 independent experiments, with n = 3 slides per treatment group.

2.4. Serum antioxidant status

Antioxidant status of serum samples from PND21 offspring of CON and 5% BB groups was evaluated by oxygen radical absorbance capacity (ORACFL) assay, which was conducted in a FLUOstar Galaxy plate reader (BMG Lab Tech, Durham, NC) as described previously [39].

2.5. Statistical analyses

Data are presented as means ± SEM. Statistical computations were performed using SigmaStat for Windows, Version 3.5 (SPSS, Chicago, Ill). Comparison of data set was performed by Student t test between 2 dietary or treatment groups and by 1-way analysis of variance followed by Tukey post hoc analyses across groups. Statistical significance was set at P < .05. Sample sizes for tissue collection were determined based on power analysis using freeware retrieved from http://www.stat.uiowa.edu/~rlenth/Power.

3. Results

3.1. Developmental and growth parameters

Body weights (P = .007) and abdominal tissue weights (P = .011) were higher in pups whose dams had 5% BB than other amounts of added BB (0%, 2.5%, and 10%) in their diets (Table 3). Blueberry at all doses did not promote sexual maturity and had no effect on uterine weights relative to the CON diet.

3.2. Mammary gland morphologic analyses

Representative carmine-stained whole mounts showed that the mammary glands of BB offspring (Fig. 2A) exhibited increased morphogenesis and were more highly branched than those of CON pups. The number of mammary branchpoints in 5% BB was higher than those of the 10% BB and CON groups (P = .027) and was comparable to the 2.5% BB group (Fig. 2B). There were no differences in mammary TEB numbers among dietary groups (Fig. 2C). However, average TEB sizes were higher for the 2.5% and 5% BB groups (P = .03) than for the CON group (Fig. 2D). Corresponding data for the 10% BB group could not be determined because of the lack of sufficient numbers of paraffin-embedded glands showing TEBs for this dietary group. Adipocyte sizes in the mammary fat pad were not altered by 5% BB relative to CON (Fig. 3A, B); a similar lack of differences in adipocyte sizes was observed among the other BB groups (data not shown).

3.3. Mammary phospho-H3 and PTEN immunoreactivity

Immunostaining of mammary sections for the mitotic indicator phospho-H3 showed nuclear staining to be significantly attenuated in mammary ductal epithelium and TEBs of BB (2.5% and 5%) relative to the CON group (Fig. 4A). Five percent BB showed greater antiproliferative effects than 2.5% BB in DE and comparable antiproliferative effects with 2.5% BB in TEB. Mammary ductal epithelium for pups of the CON and 5% BB groups showed immunostaining for PTEN in both cytoplasmic and nuclear compartments, as shown in the representative micrographs (Fig. 4B). In the nuclear compartment, the 5% BB group exhibited a modest but significant increase in PTEN immunoreactivity (P = .023) than the CON group (Fig. 4C), suggesting enhanced differentiation. Cytoplasmic immunoreactivity of PTEN was significantly lower in both the 2.5% BB and 5% BB groups (P < .05) than for the CON group (Fig. 4D). These data suggest that blueberry extract increased the differentiation of mammary epithelial and stromal compartments to a greater extent than did dietary blueberry levels of 2.5% and 5% BB, which is consistent with the greater morphologic changes seen in BB-fed pups.
PTEN immunoreactivity did not differ between the 2 dietary groups \((P = .300)\). Terminal end buds also displayed PTEN immunoreactivity; however, the percentage of PTEN-positive cells between CON and 5% BB groups did not differ (data not shown).

### 3.4. Systemic factor activity

To evaluate whether induction of PTEN immunoreactivity with 5% BB diet in vivo can be recapitulated by 5% BB serum in vitro, nontumorigenic MCF-10A cells were treated with sera (1% final concentration) from PND21 offspring exposed to control and 5% BB maternal diets. Cells treated with CON and 5% BB sera demonstrated PTEN immunoreactivity (red), although those treated with 5% BB sera showed higher PTEN localization (purple; merged images) in the nucleus (localized by DAPI stain) (Fig. 5A). The specificity of the PTEN immunoreactivity was confirmed by the loss of PTEN staining when a mixture of PTEN blocking peptide and anti-PTEN antibody was used in place of antibody alone (Fig. 5A). Cells treated with 5% BB sera showed higher nuclear immunoreactive PTEN \((P = .05)\) than those treated with CON sera (Fig. 5B). Sera from 5% BB \((17548.0 \pm 695.7 \mu mol \text{ TE per liter})\) and CON
(15524.5 ± 990.3 μmol TE per liter) groups had comparable antioxidant capacity.

4. Discussion

Regular consumption of fruits has been linked to low incidence of many types of chronic diseases. Berries are rich in anthocyanins and other polyphenols [24], and are increasingly touted for their health benefits including in the reduction of cardiovascular and cancer risks [19-21]. The signaling pathways by which dietary intake of berries and associated bioactive factors influence cell proliferation, inflammation, angiogenesis, and apoptosis for the prevention of many cancer types in rodent models have been described [40-42]; however, analogous mechanisms for the mammary cancer preventive effects of freeze-dried berries and constituents remain limited [43,44]. In this study, we showed that exposure to freeze-dried BB powders via maternal diet (in utero and lactational) enhanced mammary gland development in female prepubertal rat offspring. The dramatic changes seen in mammary architecture with 5% BB, and to a lesser extent 2.5% BB, relative to control diet were denoted by increased number of branching points and larger TEB size. Furthermore, these changes were associated with lower numbers of mitotic cells in ductal epithelium and TEBs and higher nuclear PTEN expression in ductal epithelium, in the absence of corresponding effects on TEB numbers, adipocyte size, and onset of sexual maturation. Interestingly, exposure to the highest concentration (10%) of the powdered whole BB did not affect any of the measured growth and mammary tissue parameters in offspring relative to control diet. Based on our data, we accept the hypothesis that early dietary exposure to BB via

Fig. 3. Effects of exposure to blueberry (BB) via maternal diet on mammary adipocyte size. (A) Representative hematoxylin-eosin stained sections of mammary fat pads from prepubertal rats of CON and 5%BB groups. (B) The distribution of adipocyte sizes in mammary fat pads was not altered by dietary exposure to 5% BB, relative to CON. Mean values (±SEM) are from n = 5 female pups per diet group.
maternal diet influences mammary gland development, indicating that epithelial cells from developing mammary glands are early physiologic targets of bioactive factors in BB.

In animal models of tumorigenesis or mammary tumor cell lines, previous studies have used fruit extracts and juices or purified phytochemical constituents to evaluate mechanisms underlying the antitumor activities of berries and
Fig. 5. Nuclear localization of PTEN in the non-tumorigenic human mammary epithelial cell line MCF-10A upon treatment with sera from prepubertal female rat pups of CON and 5% BB groups. Sera were used at 1% final concentration. Cells were immunostained with PTEN and counterstained with DAPI to localize nuclear compartments. Merged images show nuclear localization of PTEN (purple). As a negative control, cells were incubated with a mixture of PTEN antibody and PTEN blocking peptide (5-fold excess).

(A) Representative immunofluorescence fields showing PTEN immunoreactivity in cells treated with sera from PND21 female pups of CON and 5% BB diet groups, and in the presence of PTEN blocking peptide. (B) Nuclear PTEN-positive cells treated with sera were counted from 5 different areas per slide at 20× magnification, with 3 independent slides analyzed per treatment per experiment. The percent of nuclear PTEN-staining cells (means ± SEM, n = 2 experiments) was significantly increased (*P ≤ 0.05, by Student t-test) in those incubated with sera from 5%BB rat pups, relative to those incubated with CON sera.
associated constituents on mammary epithelial cells [23,40-48]. The present study provides the first evidence of effects of early exposure to whole blueberries (in utero to weaning) on the differentiation status of developing mammary glands. Because increased mammary differentiation (ie, increased cell cycle arrest) and higher nuclear PTEN expression are hallmarks of cellular status refractory to carcinogenic insult [12,26,30], early exposure to BB (5%) will likely be mammary tumor protective. A limitation to the current study is the lack of a demonstrated linkage between early developmental changes in the mammary gland with BB and decreased breast cancer risk. Future studies using the NMU rat model of tumorigenesis will address this question. Interestingly, although both TEBs and ductal epithelium showed progressive decreases in mitotically active cells with BB (compare 2.5% and 5% BB relative to control), only ductal epithelium from 5% BB demonstrated higher nuclear PTEN expression. The latter suggests that bioactive factor(s) in BB may mediate the reduction in DE proliferation through increased nuclear PTEN, whereas the decreased proliferation in TEB might be a PTEN-independent event.

A major question raised by our studies is the nature and identity of bioactive components in BB that may underlie the mammary branch density changes observed with early dietary exposure. Numerous studies have evaluated specific phytochemical constituents in berries for their in vitro effects on mammary epithelial cells [23,44,45,47,48]. In fractionated berry extracts, phenolic fractions are highly active in inhibiting the proliferation of breast cancer cells; these include caffeic acid, ferulic acid, and protocatechuic acid [44,47]. Because dietary exposure is indirect (ie, through the dams), the biological effects of BB likely reflect the actions of bioactive components that are able to cross the placenta and/or are present in milk. The presence of phenolic acids in maternal sera, amniotic fluids, and in milk has not been reported, and future studies to identify markers of blueberry intake in sera of dams and offspring should address this limitation in the current study. However, our analyses of urine from BB-fed rats detected significant amounts of these same phenolic acids (ferulic acid, caffeic acid, and protocatechuic acid), suggesting their presence in sera (Prior RL et al, unpublished results). The latter findings raise the possibility of fetal exposure to bioactive compounds in BB upon maternal consumption and that the biological effects noted in prepubescent mammary glands may be a consequence of neonatal responses to fetal exposure.

To provide a mechanistic perspective to the in vivo changes observed in mammary tissues with BB diet, we evaluated sera from PND21 offspring exposed to control and 5% BB via maternal diets for induction of nuclear PTEN levels and for antioxidant activity. Sera from weanling rats of the 5% BB group increased nuclear PTEN levels in MCF-10A nontumorigenic cells, an effect which recapitulated that seen in mammary ductal epithelium of 5% BB pups in vivo. The latter findings suggest lactational transfer of bioactive compounds to pups from dams consuming BB, albeit the identities of these compounds are unknown. Our observed lack of an association between dietary induction of mammary morphogenesis in vivo and increased nuclear PTEN accumulation in vitro with serum antioxidant activity is consistent with the absence of correlation between the antiproliferative activity of berry juices [43] and phenolic compounds [44] and their antioxidant capacity. The induction of nuclear PTEN localization by oxidative stress has been recently reported [49]. Thus, tissue-specific alterations in oxidative enzyme activities may be associated with increased nuclear PTEN accumulation in mammary epithelial cells with 5% BB exposure, independent of antioxidant activity in sera.

In summary, the enhanced mammary differentiation status of prepubescent female rats due to early exposure to powdered whole blueberry through maternal diet suggests that epithelial cells of the developing mammary glands are early targets of berry-associated bioactive factors acting systematically. By promoting the early transition of mammary epithelial cells from a proliferating to a functionally differentiated state, early dietary exposure to blueberry, whose bioactive components demonstrate oncoprotective activities, may increase resistance of epithelial cells to endogenous/exogenous carcinogenic insults. The possibility of altering mammary health status during early life to attenuate breast cancer risk at later adult life is consistent with a linkage between early “nutritional” experiences and development of adult diseases and provides a strong rationale for further studies to understand maternal contribution to preventing diseases that may have origins during early development.

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References


