The objective of the present study was to investigate the anti-inflammatory properties of blueberry polyphenols on MC3T3-E1 pre-osteoblasts under inflammatory condition. Cells were treated with blueberry polyphenols at concentrations of 0, 10, 100 µg/ml. After 24 h incubation, cells were stimulated by pro-inflammatory cytokines, TNF-α (10 ng/ml), IL-1 (10 ng/ml) alone or in combination for 24 h and 48 h. Cells were harvested and supernatants were collected for assessing NO production using Griess reagent. RNA was extracted and gene expression of bone turnover biomarkers was measured using cDNA array. Blueberry polyphenols were able to suppress NO production induced by either TNF-α or IL-1. However, treatment with blueberry polyphenols did not show any significant effects on cells treated with combined TNF-α and IL-1. At gene expression levels, blueberry polyphenols up-regulated RANKL and IL-6, two factors involved in osteoclast differentiation via NF-κB signaling pathway. Gene expressions of osteoblastogenic transcription factors, Runx2 and OSX, were not significantly affected by blueberry polyphenols. However, it is noteworthy that blueberry polyphenols treatment group tended to increase these osteoblastogenic factors.

INTRODUCTION

Osteoporosis is a common type of skeletal disorder characterized by low bone mineral density, which increases risk of bone fractures. Bone is a dynamic tissue that undergoes a constant remodeling process that removes old bone and generates new bone. Bone loss occurs when the activity of bone resorption by osteoclasts exceeds the activity of bone formation by osteoblasts. Previous studies have suggested that in postmenopausal women bone remodeling is greatly increased by estrogen deficiency [1]. Besides hormonal regulations in bone homeostasis, some studies have shown the effects of pro-inflammatory cytokines in explaining the pathogenesis of osteoporosis in aging. These studies have shown that pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), are produced during aging, presenting positive relationship with the incidence of bone loss and osteoporotic fracture in both men and women of older age [2].

Although pharmaceutical therapy can somehow prevent or treat osteoporosis, the high cost of medicine and its adverse effects [3] encourage people to find alternative therapies to treat osteoporosis. In recent years, cell culture studies, animal studies, and human clinical trials suggest that phytochemicals, especially polyphenols, present in fruits and vegetables, might be potential candidates for substituting conventional medicine for osteoporosis [1, 4, 5]. However, the capacity of maintaining bone health by polyphenols varies, which might result from the different contents and quantity of polyphenols in different fruits and vegetables.

Blueberry (BB) is a rich source of phytochemicals, containing phenolic acids (e.g. Gallic acid, coffee, ferule and pelagic acids) and flavonoids (anthocyanins, catching and quercetin) [6]. According to USDA report BB possesses the highest antioxidant activity among all the selected foods [7]. This concept has attracted researchers to explore the protective effects of BB on chronic diseases triggered by oxidation and inflammation, such as colon cancer [8], insulin resistance [9], and poor lipid profiles [10]. Our previous study showed that 5% BB treatment (w/w) for 100 days was able to prevent the loss of whole-body bone...
mineral density induced by ovariectomy (OVX) in rats as well as suppressing femoral mRNA levels of bone turnover biomarkers (alkaline phosphatase, collagen type 1 and tartrate-resistant acid phosphatase) that were elevated by estrogen deficiency [11]. Because of the known high content of antioxidant and anti-inflammatory compounds of the BB as well as the results from a previous study suggesting BB has beneficial effect on inflammation-related diseases, we hypothesized that BB polyphenols possess anti-inflammatory properties that reduces inflammation markers in inflammatory-induced pre-osteoblast cells (MC3T3-E1). Therefore, the present study was designed to examine the extent to which BB polyphenols prevents inflammation in TNF-α and/or IL-1-induced MC3T3-E1 cells.

MATERIALS AND METHODS

Extraction of dried BB polyphenols

Dried BB powder was generously donated by U.S. Highbush Blueberry Council (Folsom, CA, USA). Ten grams of dried BB powder was dissolved in 100.0 ml of 80% ethanol and sonicated for 20 minutes at room temperature under subdued light with purging nitrogen gas. The solution was filtered through filter paper (No. 2 Whatman, Clifton, NJ) under a vacuum suction. The remaining residue was rinsed with 50.0 ml of 100% ethanol, and sonicated again. Filtered extracts were concentrated on the rotary evaporator (Bochi R-3000, Flawil, Switzerland) until there was no ethanol distilled from the evaporator and finally freeze dried (BenchTop K, Virtis, Gardiner, NY).

Cell culture

MC3T3-E1 pre-osteoblast-like cells were purchased from ATCC (Rockville, MD). Cells were cultured in α-modified minimal essential medium (α-MEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37ºC in a 5% CO2 humidified incubator. The medium was changed at three-day intervals.

Cell viability assay

The cell viability was measured by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay (MTT Assay, ATCC, Rockville, MD), which detected metabolic conversion of MTT to formazan by living cells. MC3T3-E1 pre-osteoblasts were seeded in 96-well plates at a density of 1×104 cells per well in the presence of increasing doses of BB polyphenol extracts (0, 10, 100 µg/ml). After 24 h, cells were treated with either TNF-α (10 ng/ml) or IL-1 (10 ng/ml) alone or combined for 24 h. Total cellular RNA was isolated from pre-osteoblast culture using Trizol reagent (Invitrogen, Rockville, MD) according to the manufacturer’s instruction. The concentration of mRNA was read and calculated by spectrophotometer (Ultrospec 2100 pro, GE Healthcare, Piscataway, NJ) and RNase-free H2O was used as a blank. Purification of samples was tested by agarose gel (1%) electrophoresis. Denatured RNA (5.5 µg) from cells were reverse transcribed with mouse custom primer mix (Signosis Biosignal Capture, Sunnyvale, CA) under the following conditions: 65 ºC for 5 min, 45 ºC for 1 h, and 98 ºC for 5 min. Then the 20 µl of synthesized cDNAs was hybridized to gene-specific oligonucleotide precoated in the plate, TNF-α tumor necrosis factor receptor-2 (TNFR2), TNF receptor-associated factor (TRAF), nuclear factor kappaB ligand (RANKL), macrophage-colony stimulating factor (M-CSF), IL-1, IL-6, nitric oxide synthase-2 (NOS2), cyclooxygenase-2 (COX-2), bone morphogenic protein-2 (BMP-2), osterix (OSX), runt-related transcription factor-2 (RUNX2), and osteoprotogerin (OPG). The captured cDNAs were later detected with streptavidin-HRP. The expression level of genes was proportional to the luminescent intensity obtained from luminometer SpectraMax M5 (Molecular Devices, Sunnyvale, CA) reported as relative light units (RLUs). The final data were normalized to the housekeeping gene 18s rRNA and compared to control group.

Statistical analysis

The data are presented as mean ± SD. To assess statistical significance, values were compared using analysis of variance (ANOVA). When ANOVA indicated statistical significance, Tukey’s post hoc multiple comparisons test was used to determine that means were significantly different. SPSS software version 16.0 (Somers, NY, USA) was used for all statistical analyses. Significance level was accepted at P<0.05.

RESULTS AND DISCUSSION

Cell viability

Cell viability decreased in cells treated with TNF-α alone or TNF-α and IL-1 combined compared to the control group after 24 h. IL-1 alone had no effect on cell viability after 24 h. BBP had no obvious effects on cell viability (Figure 1).

TNF-α alone or combined with IL-1 for 48 h significantly (P<0.05) decreased cell viability by 30% and 45% compared.
to the control group, respectively; while treatment with IL-1 alone for 48h did not affect cells viability. (BBP) did not have a significant effect on cell viability treated with TNF-α or IL-1 alone (data not shown). However, there was a slight increase in cell viability when cells treated with TNF-α and IL-1 combined.

**Nitric oxide production**

The function of NO in bone metabolism is controversial among in vivo and in vitro studies. It has been shown that NO accumulation is protective against bone loss in OVX rats [12]. However, NO inhibited alkaline phosphatase (ALP) activity and promoted production of prostaglandin 2 in MC3T3-E1 cells, suggesting that NO is capable of suppressing osteoblastic function and stimulating osteoclastic function [13]. Nitric oxide is synthesized from L-arginine by NO synthase (NOS). There are three NOS: endothelial NOS, constitutive NOS and NOS2. Production of NOS2 is stimulated by the presence of cytokines, such as TNF-α and IL-1 [13].

Treatment with either TNF-α (10 ng/ml) or IL-1 (10 ng/ml) stimulated NO production in MC3T3-E1 pre-osteoblasts 11.7 and 9.97 folds, respectively. Nitric oxide concentration was further increased in the presence of TNF-α and IL-1 combined. Treatment with BBP was able to suppress the NO production induced by TNF-α or IL-1 alone. However, BBP had no significant effect on reducing the NO levels stimulated by TNF-α and IL-1 combined (Figure 2).

Treatment with TNF-α or IL-1 alone for 48h had no effect on NO production. However, treatment with TNF-α and IL-1 combined triggered cells to produce NO in extremely high levels, 66.55 folds higher than the control group. Blueberry polyphenol had no effect on NO production after 48 h stimulation with TNF-α and IL-1 alone or in combination (Data not shown). These results are consistent with the results reported by Kanematsu et al. [13]; however, in another study by Suh et al. [14] no significant NO production was detected in osteoblastic cell media in the presence of TNF-α. Furthermore, our findings showed that the gene expression of NOS2 that is regulated by TNF-α and IL-1 was similar to that of NO production. However, BB polyphenols did not have significant effect on regulating NOS2 gene expression.

**Gene expression of bone turnover regulators**

RANKL is a member of TNF superfamily derived from OC precursors, mature OC and dendritic cells. Once RANKL binds to its receptor RANK, the signal is sensed by TNF-α receptor associated factors (TRAFs) and then transduced to NF-κB to stimulate production of molecules that are essential for differentiation and maturation of pre-osteoclasts [15,16]. Treatment of pre-osteoblast cells with TNF-α, IL-1 or the combination of both cytokines significantly increased the RNA expression of RANKL. These results are consistent with other studies that showed TNF-α can synergize with RANKL to promote osteoclastogenesis [17]. However, BB polyphenols had no significant effect on TNF-α induced gene expression level of RANKL in the present study.

In addition, TNF-α elevated IL-6 and NOS2 RNA expression levels while IL-1 increased TNFR2 RNA expression levels (Table 1). TNF-α-induced elevated gene expressions of IL-6 and NOS2 were significantly further up regulated by BB polyphenols treatment. Gene expressions of TRAF, NF-kB and NOS2, which were not promoted by the combination of TNF-α and IL-1, were significantly elevated by BB polyphenol treatment. There were no difference among all the treatments with respect to the RNA expression levels of OSX, RUNX2 and OPG, factors important for regulating differentiation and activation of pre-osteoblasts (Table 1). Also, BB polyphenols treatment did not have any effects on preventing apoptosis resulted from TNF-α stimulation.

IL-1 is another important pro-inflammatory cytokine involved in pathogenesis of inflammation-induced osteoporosis by stimulating other pro-inflammatory cytokines, such as IL-6 and IL-11 that facilitates differentiation of OC progenitors [18]. Thus far, the mechanisms by which IL-1 regulates bone turnover are...
Figure 2. Effects of BB on NO production of MC3T3-E1 pre-osteoblasts. Cells were treated with grading doses of blueberry polyphenol extracts (0, 10, 100 μg/ml) for 24 h and then treated with TNF-α (10 ng/ml), IL-1 (10 ng/ml), or combination for 24 h. The NO production was measured by Griess reagent and was represented as mean ± SD in the bars. Different letters indicate significant different values at P<0.05 (n=6).

Table 1: Effects of BB on gene expressions of osteoclastogenic and osteoblastogenic factors. Cells were treated with BB (10 μg/ml) for 24 h, and then treated with TNF-α (10 ng/ml) or IL-1 (10 ng/ml) or mixture for 24 h.

<table>
<thead>
<tr>
<th>Factors</th>
<th>Control</th>
<th>TNF-α (10 ng/ml) + blueberry (10 μg/ml)</th>
<th>IL-1 (10 ng/ml) + blueberry (10 μg/ml)</th>
<th>TNF-α+IL-1 + blueberry</th>
<th>TNF-α+IL-1</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Osteoclastogenesis (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>3.84 ± 0.50</td>
<td>404.64 ± 364.47</td>
<td>418.44 ± 260.22</td>
<td>441.83 ± 292.80</td>
<td>326.38</td>
<td>0.183</td>
</tr>
<tr>
<td>TNFR2</td>
<td>0.59 ± 0.20a</td>
<td>18.49 ± 3.60a</td>
<td>74.79 ± 47.23</td>
<td>38.86 ± 25.87a</td>
<td>29.06 ±</td>
<td>0.031</td>
</tr>
<tr>
<td>TRAF</td>
<td>0.005 ± 0.002a</td>
<td>10.89 ± 10.50a</td>
<td>21.64 ± 10.32a</td>
<td>21.64 ± 13.47a</td>
<td>7.94 ±</td>
<td>0.021</td>
</tr>
<tr>
<td>NF-kB</td>
<td>0.001 ± 0.002b</td>
<td>12.78 ± 0.06a</td>
<td>32.37 ± 38.49a</td>
<td>20.71 ± 9.37a</td>
<td>15.71 ±</td>
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<td>RANKL</td>
<td>30.66 ± 5.0a</td>
<td>1357.50 ± 795.77b</td>
<td>1253.80 ± 346.89b</td>
<td>1047.40 ± 572.90b</td>
<td>786.60 ±</td>
<td>0.029</td>
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<tr>
<td>M-CSF</td>
<td>4.34 ± 0.50</td>
<td>153.39 ± 68.97</td>
<td>185.07 ± 172.00</td>
<td>88.14 ± 56.86</td>
<td>103.95 ±</td>
<td>0.184</td>
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<tr>
<td>IL-1</td>
<td>0.043 ± 0.02</td>
<td>36.25 ± 19.34</td>
<td>42.19 ± 20.92</td>
<td>34.06 ± 21.48</td>
<td>16.29 ±</td>
<td>0.191</td>
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<tr>
<td>IL-6</td>
<td>0.401 ± 0.051</td>
<td>27.60 ± 19.18b</td>
<td>57.61 ± 32.32b</td>
<td>19.64 ± 10.009bc</td>
<td>13.22 ±</td>
<td>0.013</td>
</tr>
<tr>
<td>NOS2</td>
<td>10.157 ± 0.51a</td>
<td>142.7 ± 34.03a</td>
<td>198.71 ± 120.17b</td>
<td>74.31 ± 37.08bc</td>
<td>70.07 ±</td>
<td>0.007</td>
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<tr>
<td>COX2</td>
<td>0.001 ± 0.002</td>
<td>8.52 ± 8.15</td>
<td>5.48 ± 2.10</td>
<td>16.47 ± 17.12</td>
<td>11.76 ±</td>
<td>0.220</td>
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<tr>
<td>Osteoblastogenesis (100%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>BMP2</td>
<td>0.03 ± 0.001</td>
<td>7.40 ± 1.98</td>
<td>25.04 ± 6.96</td>
<td>14.41 ± 19.46</td>
<td>8.08 ±</td>
<td>0.138</td>
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<tr>
<td>OSX</td>
<td>0.004 ± 0.001</td>
<td>13.32 ± 2.85</td>
<td>15.97 ± 20.01</td>
<td>10.44 ± 16.61</td>
<td>3.27 ±</td>
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<td>RUNX2</td>
<td>49.87 ± 5.00</td>
<td>1537.70 ± 760.45</td>
<td>1354.00 ± 743.28</td>
<td>1333.70 ± 983.02</td>
<td>979.59 ±</td>
<td>0.105</td>
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<tr>
<td>OPG</td>
<td>0.030 ± 0.001</td>
<td>17.42 ± 2.54</td>
<td>16.45 ± 18.22</td>
<td>6.45 ± 4.05</td>
<td>11.82 ±</td>
<td>0.092</td>
</tr>
</tbody>
</table>

The level of gene expression was normalized by internal control 18s rRNA and expressed as RULs. Values are presented as means ± SD. Different letters indicate significant different values at P<0.05 (n=3).

Still unclear. Some studies indicate that IL-1 has positive effects on mature OC in a RANKL-independent pathway [19], while other studies showed that IL-1 failed to exert osteoclastogenesis effects in the absence of RANKL [20]. Our findings demonstrated that gene expressions of TNFR2 and RANKL were increased significantly in IL-1 treated group. Also, gene expressions of TRAF and NF-κB tended to increase in cells treated with IL-1. Therefore, we speculated that IL-1 might synergize with both TNF-α and RANKL to activate pre-osteoclasts, which supports the latter perspective aforementioned.

CONCLUSION

In conclusion, this is the first study that attempts to identify the mechanism by which BB polyphenols act on inflammation.
induced MC3T3-E1 pre-osteoblasts. The results indicated that pro-inflammatory cytokines, TNF-α and IL-1 stimulate NO production, which was significantly reduced by BB polyphenols treatment. Also, the mRNA expression of transcription factors and cytokines, RANKL, TRAF2, IL-6, that are important for initiating osteoclastogenesis, increased significantly. Whole polyphenol extract from frozen BB powder was used in the present study. Future studies will further explore whether the whole fruit or the most efficacy component of the blueberry polyphenol can exert the protective effects on inflammation-induced osteoporosis.

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7. USDA. Oxygen Radical Absorbance Capacity (ORAC) of Selected Foods. 2007.


