BERRY QUALITY

RESEARCH

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16. TITLE: Summary of Relevant Findings on the Health Benefits of Wild Blueberry

OBJECTIVES

The objectives of this study were to determine the effect of Anthocyanins (ACNs), Phenolic Acids (PAs) and their combinations extracted from wild blueberries: **a.** on the speed of endothelial cell migration and angiogenesis (new blood cell formation) after exposure to the above fractions and **b.** changes in gene expression and concentrations of protein levels critical for cell migration and angiogenesis (RHO, RAC1, AKT1, VEGF, eNOS).
LOCATION: Dr. Klimis-Zacas Laboratory, Hitchner Hall University of Maine, Orono
PROJECT TIMEFRAME: September 2016- September 2019

INTRODUCTION

The wound healing process is critical in conditions such as diabetic wounds, burns, blast injuries, ulcus decubitus, skin regeneration etc, and involves many cell types, including endothelial cells, that when stimulated, they proliferate, migrate and promote angiogenesis and tissue remodeling leading to wound healing (Guo & Dipietro 2010; Kolluru, Bir & Kevil 2012). Chronic diseases such as diabetes lead to impaired wound healing, a result of impaired angiogenesis (new blood vessel formation) (Abaci et al. 1999; Kolluru et al. 2012; Waltenberger, Lange, & Kranz 2000) which is due to reduced blood flow to the extremities, decrease in endothelial cell proliferation, migration and angiogenesis (Kolluru et al. 2012).

The lowbush blueberry (*Vaccinium angustifolium)* is one of the richest food sources of bioactive compounds (polyphenols) such as anthocyanins (ACNs) and phenolic acids (PAs) (Del Bo, Cao, et al. 2016). The antioxidant activity of wild blueberries is a result of ACNs, procyanidins, chlorogenic acid, and other phenolic compounds (Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor 2015).

Numerous *in vivo* and *in vitro* studies in our laboratory and others have documented the beneficial effects of wild blueberry consumption (diets) on Obesity-induced inflammation, Cardiovascular Disease (CVD) as well as many other chronic diseases (Del Bo, Cao, et al. 2016; Del Bo et al. 2010; Del Bo, Roursgaard, et al. 2016; Kay & Holub 2002; Kristo, Kalea, Schuschke & Klimis-Zacas 2010; Riso et al. 2013; Skrovankova et al. 2015; Vendrame, Kristo, Schuschke & Klimis-Zacas 2014). There is paucity of studies though on single ACNs and PAs extracted from wild blueberries on cell migration, angiogenesis and wound healing.

METHODS

Cell Culture

Human umbilical vein endothelial cells (HUV-EC-C [HUVEC] (ATCC® CRL-1730™)) were used.

Extraction and Analysis of ACNs and PA Fractions from Wild Blueberry Powder

Wild blueberries (WB) were provided as a composite by Wyman's (Cherryfield, Maine, USA) and processed following standard procedures to obtain a freeze-dried powder (FutureCeuticals, Momence, Ill., USA) (Vendrame et al. 2014). From the freeze-dried wild blueberry powder three

fractions were isolated: **1.** Phenolic-rich fraction (ethyl acetate soluble, containing mainly chlorogenic acid) **2.** Anthocyanin-rich fraction (methanol soluble fraction, containing mainly anthocyanins) and **3.** Water soluble fraction. The concentration of ACNs and PAs in the wild blueberry fraction was determined by HPLC.

Cell Proliferation and Cytotoxicity Assay

The cell growth curve experiment provided information on the doubling time of the HUVECs. The ACNs and PAs cytotoxicity assay was conducted so that the **optimum concentration and exposure time** of the active compounds was used for the rest of the experiments. Cell proliferation and cytotoxicity assays were conducted by using the alamar Blue assay (Life Technologies, DAL1025).

Cell Migration

The effect of ACNs, PAs and their combination on cell migration was evaluated by the IBIDI Culture–Insert (Ibidi, Munich, Germany). They were treated with the PA and ACN fractions and their combination at concentrations as determined by the results of the cytotoxicity experiment (0.002 µg/mL, 8 µg/mL, 15 µg/mL, 60 µg/mL and 120 µg/mL). Cells in the treated (ACN and PA fractions and combination) and untreated (control) wells were observed under an inverted phase-contrast optical microscope (Nikon TS100) until endothelial cells fully migrated into the free area. At the end of each experiment the speed of closure was calculated as the cell migration rate ($v_{migration}$ in µm/hr). Analysis was conducted with the TScratch software as described in detail by Geback and Jonkman (Geback, Schulz, Koumoutsakos & Detmar 2009; Jonkman et al. 2014).

Gene Expression, Real-time RT-PCR Analysis

Endothelial cells were cultured and maintained as described previously. HUVECs were treated with ACN and PA fractions and their combination for 2 h and 6 h. mRNA was isolated using the RNeasy Kit (Qiagen) and DNase Digestion (Qiagen) was used for RNA purification. QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis and removal of genomic DNA.

Active GTPase Pull-down Assay

For immunoprecipitation, cells were lysed using the Cell Signaling active RHO detection kit (8820) and active RAC1 detection kit (8815) following manufacturer's instructions and treated with GTPγS (positive control) and GDP (negative control).

Angiogenesis Assay (tube formation)

Endothelial cells ($1x10^4$ cells/well) were plated and cultured on Matrigel (BD Biosciences) and were applied on an IBIDI µ-slide Angiogenesis plate (Ibidi, Martinsried, Germany) and incubated at 37 °C for 30min for gel construction thus cells were induced to form capillary-like tubes. After exposing the cells to specific concentrations of anthocyanin, phenolics and combinations of both fractions, including a control (untreated cells), the effect on the tube formation was photographed by using an inverted phase-contrast optical microscope (Nikon, TS100) 4h after exposure, based on the treatment and number of meshes, nodes and master junctions, total mesh area and total master segment length was measured and analyzed with the computer program Image J with the Angiogenesis Analyzer plugin. Western Blot Analysis was used for the for the detection of AKT1, VEGF, and eNOS.

RESULTS

Our results document for the first time, that treatment of human endothelial cells with ACNs at 60 µg/mL inhibit endothelial cell migration rate while treatment with PAs at 0.002 µg/mL, 60 µg/mL and 120 µg/mL significantly increase endothelial cell migration rate compared to control. Moreover, exposure of HUVECs to ACNs:PAs at 8 µg/mL:8 µg/mL and 60 µg/mL:60 µg/mL significantly increase endothelial cell migration. Gene expression of RAC1 and RHOA, critical

proteins for cell migration, significantly increased 2 h after exposure with all treatments. Findings suggest that endothelial cell migration is differentially modulated based on the type of blueberry extract (ACN or PA fraction) and is concentration dependent. Below, please find a schematic summary of the already published results (Tsakiroglou et al. 2019).

Angiogenesis Experiment

Treatment with ACNs decreased endothelial cell tube formation as well as downregulated gene expression of AKT1 and eNOS, while treatment with PAs and ACNs:PAs increased overall endothelial cell tube formation and upregulated AKT1 and VEGF gene expression compared to control. Findings suggest that angiogenesis is significantly promoted by the PA and ACN:PA fraction treatment, especially important for promotion of wound healing. These are novel findings and have not been documented before the present time**.** *A manuscript is presently under review so actual results cannot be shown here.*

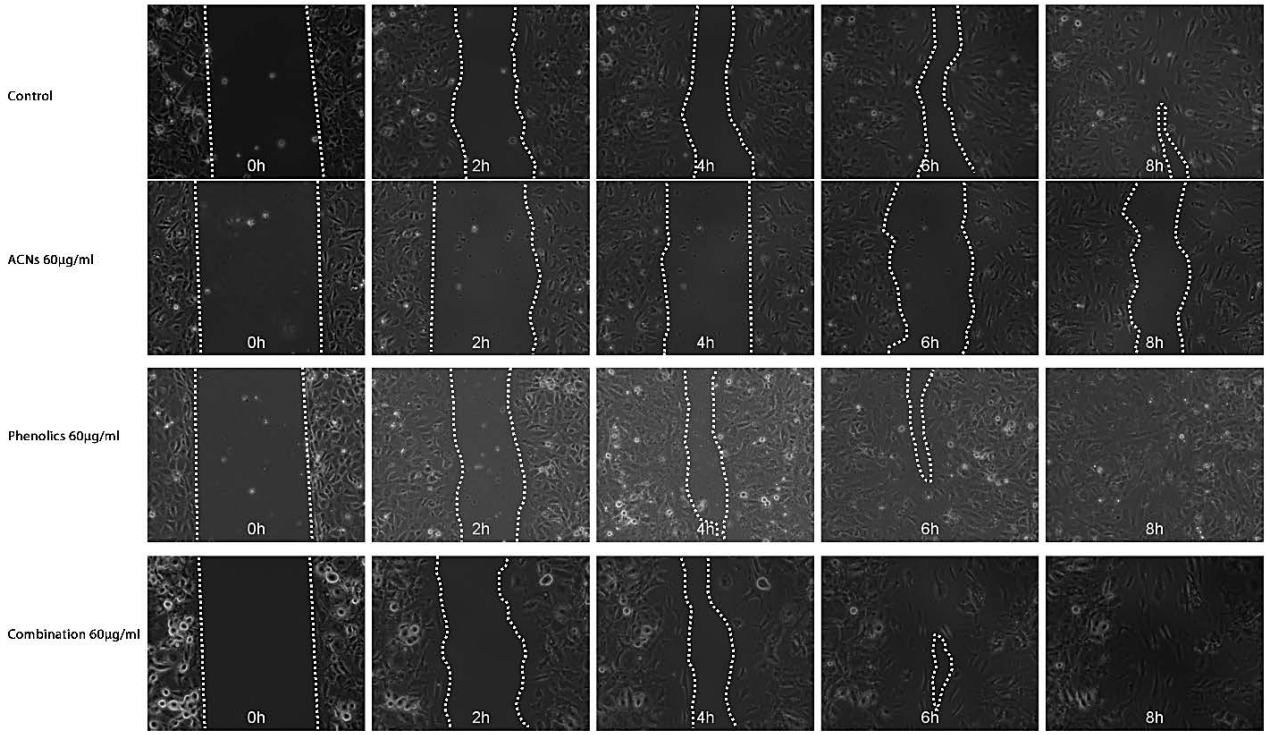

Figure 1. Summary of speed of endothelial cell migration after exposure to ACNs at 60 µg/mL, PAs 60 µg/mL and ACNs:PAs 60 µg/mL:60 µg/mL. Frames are from time-lapse video at 0 h, 2 h, 4 h, 6 h and 8 hs after exposure.

DISCUSSION

Thus, we document for the first time that different fractions extracted from wild blueberries have a significant and differential effect on endothelial cell migration and angiogenesis which plays a key role on many physiological phenomena such as wound healing. These differential effects seem to be dose and compound dependent and be orchestrated by the modulation of gene expression of RAC1 and RHOA, AKT1, eNOS and VEGF proteins involved in cell motility and angiogenesis. Results document that Anthocyanins slow down cell migration and angiogenesis, while Phenolic acids speed up cell migration and angiogenesis. These are novel findings that to our knowledge have not been previously reported and are critical for discovering new treatments

that contain natural compounds for conditions that benefit from promoting wound healing and tissue regeneration.

CURRENT RECOMMENDATIONS

At this time, preclinical studies are underway to validate the results documented with the *in vitro* studies (cell culture). Additionally, with collaboration and funding from the Maine Technology Institute, the Office of Innovation and Economic Development of the University of Maine, the UMaine Medicine Initiative and Industrial partners, a medical prototype with possible commercialization is being developed to enhance wound healing and tissue regeneration.

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