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Extract of acai-berry inhibits osteoclast differentiation and activity

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ABSTRACT

Osteoclastogenesis is the major cellular event responsible for bone loss and is triggered by inflammation. Acai-berry has proven anti-inflammatory effects. However, there is a lack of evidence for its effects on osteoclastogenesis. Thus, the aim of this study was to determine whether acai-berry extract (ABE) could inhibit osteoclastogenesis and osteoclast activity *in vitro*. The secretion of cytokines by osteoclasts has been also evaluated. RAW 264.7 cells were stimulated with RANKL (50 ng/mL) and treated with various concentrations of ABE (25–100 μg/mL) to verify: cell viability (MTT), total protein concentration (BCA), osteoclast differentiation and activity, and cytokine secretion. Cell viability and protein assays showed no toxicity to RAW cells for the tested ABE concentrations ($p > 0.05$). ABE also showed a dose-dependent inhibition of osteoclastogenesis and osteoclast activity evaluated by tartrate-resistant acid phosphatase (TRAP) and hydroxylapatite resorption assay, respectively ($p < 0.05$). ABE decreased the secretion of interleukin (IL)-1α, -6 and tumor necrosis factor alpha while increasing the secretion of IL-3, -4, -13 and interferon gamma when compared to the control group ($p < 0.05$). Results of this study showed that acai-berry extract inhibits osteoclast differentiation and activity possibly due to the modulation of a vast number of cytokines produced by osteoclast precursor cells.

1. Introduction

Inflammatory bone diseases such as periodontal disease are triggered by chronic inflammation causing the upregulation of osteoclastogenesis. This in turn shifts the bone remodeling process toward increased bone resorption (Boyle, Simonet, & Lacey, 2003). Inflammatory cytokines have been associated with bone destruction, having a role in the regulation of the expression of the receptor activator of nuclear factor kappa B (RANK) and receptor activator of nuclear factor kappa B ligand (RANKL) which is a vital step in the activation of osteoclastogenesis (Hsu et al., 1999; Singh, Mehdi, Srivastava, & Verma, 2012).

Indeed, osteoclastogenesis can be upregulated by cytokines such as IL-1α, IL-6, and tumor necrosis factor alpha (TNFα) through activation of direct or indirect osteoclastogenesis pathways while other cytokines such as interferon gamma (IFNγ), IL-3, IL-4, IL-10 and IL-13 have shown the opposite effect by hindering osteoclastogenesis (Palmqvist, Persson, Conaway, & Lerner, 2002; Sabokbar, Kudo, & Athanasou, 2003; Singh et al., 2012).

Cells under inflammatory conditions can produce such cytokines as IL-1β and TNFα which cause additional inflammation via the activation of the NF-κB pathway (Lee et al., 2014). Cytokines have also been shown to directly affect osteoclastogenesis *in vitro*. IL-3 exhibits inhibitory actions on hematopoietic osteoclast precursor cells induced to differentiate with RANKL and MCSF. IL-3 suppresses both the NF-κB signaling characteristic of RANKL activation and also the expression of c-Fos, thus inhibiting both inflammatory pathways (Hong et al., 2013; Johnson, Spiegelman, & Papaioannou, 1992; Khapli, Mangashetti, Yogesha, & Wani, 2003). Aside from cytokines and growth factors, osteoclast differentiation and activity can also be influenced by natural compounds (Leotoing et al., 2013).

Growing evidence shows that natural compounds can inhibit osteoclastogenesis and osteoclast resorptive activity (Bu et al., 2008; Crasto et al., 2013). Amidst the many studies pertaining to the health benefits of natural compounds, polyphenols exhibit promising therapeutic potential. Polyphenols are effective as antioxidants because they increase the bioavailability of nitric oxide by providing vasodilatory and anti-hypertensive properties.

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Recent studies have also shown that polyphenols can regulate osteoclastogenesis (Bu et al., 2008). A possible explanation of the potential of polyphenols to inhibit bone loss is by inhibiting inflammatory cell signaling pathways such as NF-κB (Park & Min do, 2011). Jungbauer and Medjakovic (2012) have stated that polyphenols act on many targets and do so through receptors and eventually signal transduction or by affecting transcription pathways (Jungbauer & Medjakovic, 2012). For example, the polyphenol fisetin has been shown to have an inhibitory effect on the NF-κB pathway in macrophages induced with lipopolysaccharide (LPS). Fisetin was also seen to inhibit osteoclast differentiation in RANKL-induced osteoclastogenesis by affecting the NF-κB, p38 and JNK pathways (Leotoing et al., 2013).

Acai-berry extract is known for the abundance of polyphenols in its composition such as anthocyanins, epicatechin, velutin and catechin (Xie et al., 2011, 2012). Acai-berry, the fruit of the Amazonian palm *Euterpe oleracea* Mart., has received much attention in recent years due to its potential anti-oxidant and anti-inflammatory properties (Havsteen, 2002; Moura et al., 2012). A diet containing 5% of freeze-dried acai juice powder has also been shown to attenuate atherosclerosis in apolipoprotein E-deficient mice (Xie et al., 2011). Interestingly, the levels of some pro-inflammatory cytokines such as TNFα and IL-6 were significantly decreased in the sera and in the residential macrophages from these mice fed with this acai diet (Xie et al., 2011).

There is a lack of information about the effects of acai-berry extract on bone cells *in vitro*. It is therefore important to fill the gap in knowledge by verifying if acai-berry could also modulate the levels of inflammatory cytokines related to osteoclastogenesis and therefore regulate osteoclast formation and activity. Thus, our study aimed to determine the effect of acai-berry extract on osteoclastogenesis and osteoclast activity and cytokine secretion in RANKL-induced osteoclast precursor cells.

2. Materials and methods

2.1. Acai extract (E. oleracea *Mart.) dilutions*

Dry acai-berry extract (ABE) consisted of 99.8% of pure, finely milled acai-berry pulp powder with no binders or fillers (Embrafarma, Ceara, Brazil). The powder was suspended in DMSO (≥99.9%, Sigma-Aldrich, St. Louis, MO, USA) (100 mg/mL). After incubating for 5 min, the suspension was centrifuged and the supernatant was filtered (0.22 μm filter, Millipore, Billerica, MA, USA) in order to achieve final concentrations of 25, 50 or 100 μg/mL. Stock dilutions were kept in −20 °C during the experimental phase.

2.2. Cell culture

RAW 264.7 mouse monocyte macrophage cell line was used as an osteoclast precursor. The cells were seeded (5×10^6 cells/well) in culture flasks (75 mL BD Falcon, Franklin Lakes, NJ, USA) containing Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) and supplemented with 10% Fetal Bovine Serum (FBS), 100 μg/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 °C with 5% CO_2.

2.3. Osteoclast differentiation

After 3 days of cell growth, the RAW 264.7 cells were resuspended in α-MEM (Minimum Essential Medium Eagle, Alpha Modification, Life Technologies, Carlsbad, CA, USA) and supplemented with 10% FBS, 2 mM L-glutamine and 100 μg/mL penicillin/streptomycin, and transferred to 96-well culture plates (5×10^3 cells/well). After overnight incubation, 50 ng/mL of RANKL (Sigma-Aldrich) was added to each well, in order to induce cell differentiation (*i.e.* osteoclastogenesis). At this time, the tested dilutions of ABE (25–100 μg/mL) were added to the wells for 5 days with change of medium on day 3. Untreated cells (without ABE) were used as control.

2.4. Cell proliferation assays (toxicity and total protein)

To determine the toxicity of ABE serial dilutions from 3.125 to 400 μg/mL, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche-Mannheim, Mannheim, Germany) was used to measure the proliferation of the RAW 264.7 (n = 3). Briefly, the RAW cells were incubated for 5 days in a 96-well plate. After that, 10 μL of MTT labeling reagent was added to each well, and incubated for 4 h. The solubilisation solution (100 μL) was added into each well, and the plates were left for overnight incubation. Absorbance of the formazan was measured using a microplate (BIO RAD 3550, Hercules, CA, USA) reader. The wavelength to measure absorbance was between 550 and 600 nm.

A protein assay was used to evaluate cell proliferation (n = 3). Cells were grown in 96-well plates in presence and absence of ABE for 5 days. Cell lysis was carried out as listed in Kartner et al. (2010). Briefly cells were washed with PBS and lysed with protein lysis buffer (90 mM trisodium citrate, 10 mM NaCl, 0.1% Triton X-100, pH 4.8). Total protein concentrations were determined using the Pierce bicinchoninic acid (BCA) assay (Thermo Scientific 23225, Waltham, MA, USA) at 562 nm.

2.5. Tartrate-resistant acid phosphatase (TRAP)

RANK-induced RAW cells were differentiated into osteoclasts for 5 days in 96-well plates and treated with ABE (25–100 μg/mL) concentrations. Qualitative and quantitative measurements of TRAP were obtained using two methods in order to evaluate the effect of ABE on osteoclast differentiation (n = 3). The first was a histological TRAP stain (BD BioSciences #445, Franklin Lakes, NJ, USA) for fixed osteoclasts that were divided into two

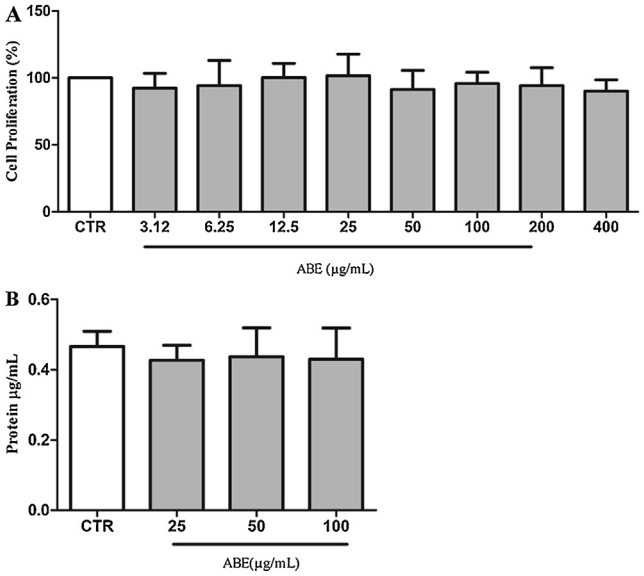

Fig. 1. ABE is not toxic to RANKL-induced RAW 264.7 cell. Cells were cultured in the presence of ABE serial dilution (3.12–400 μg/mL): (A) after 5 days of continuous ABE exposure, cell metabolic activity was assessed using MTT tetrazolium dye assay. Differences were not significant when compared to control (CTR); (B) under the same growth condition, total protein levels were unaffected at up to 100 μg/mL of ABE.