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Bioaccessible (poly)phenol metabolites from raspberry protect neural cells from oxidative stress and attenuate microglia activation

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ABSTRACT

Neuroinflammation is an integral part of the neurodegeneration process inherent to several aging dysfunctions. Within the central nervous system, microglia are the effective immune cells, responsible for neuroinflammatory responses. In this study, raspberries were subjected to *in vitro* digestion simulation to obtain the components that result from the gastrointestinal (GI) conditions, which would be bioaccessible and available for blood uptake. Both the original raspberry extract and the gastrointestinal bioaccessible (GIB) fraction protected neuronal and microglia cells against H_2O_2-induced oxidative stress and lipopolysaccharide (LPS)-induced inflammation, at low concentrations. Furthermore, this neuroprotective capacity was independent of intracellular ROS scavenging mechanisms. We show for the first time that raspberry metabolites present in the GIB fraction significantly inhibited microglial pro-inflammatory activation by LPS, through the inhibition of Iba1 expression, TNF-α release and NO production. Altogether, this study reveals that raspberry polyphenols may present a dietary route to the retardation or amelioration of neurodegenerative-related dysfunctions.

1. Introduction

The increase in life expectancy that has occurred in recent years is increasingly becoming accompanied by the intensification of neurodegenerative diseases. These diseases are characterized by an irreversible loss of brain function that may lead to cognitive deficits, dementia, movement disorders, behavior deviations and psychological disorders. Underlying pathogenic mechanisms involved in such processes include mitochondrial dysfunction, neuroinflammation, protein aggregation, defective axonal transport, excitotoxicity and oxidative stress. In the mammalian brain, reactive oxygen species (ROS) mainly superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$) are continuously being produced (Gandhi & Abramov, 2012). Generation of ROS is counteracted by effective enzymatic and non-enzymatic antioxidant systems, such as superoxide dismutase, glutathione peroxidase, catalase and glutathione. Oxidative stress may result either from an increase in ROS production, a decrease in antioxidant systems, or both, and is an important player in the aging brain and in the development of degenerative diseases (Chen, Guo, & Kong, 2012).

Neuroinflammation is mediated by glial cells and intimately entwined with the neurodegeneration process. Microglia is the major innate immune cells of the central nervous system (CNS) (Cherry, Olschowka, & O'Banion, 2014) and, in non-pathological conditions, continuously survey the surrounding environment. Upon noxious injuries, such as pathogens, endotoxins, tissue damage or dying neurons, surveilling microglial cells are activated into a pro-inflammatory M1 phenotype, also designated as the

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classical activation state (Orihuela, McPherson, & Harry, 2015). This phenotype initiates inflammatory responses mediated by the release of pro-inflammatory molecules, such as cytokines, chemokines and ROS (Cherry et al., 2014). Oxidative stress is a typical hallmark of this type of microglial activation, whether as a cause or a consequence. Microglia, as other macrophage cells, naturally have the ability to switch toward an anti-inflammatory phenotype M2, also known as the alternative activation state. This type of activation includes the inhibition of pro-inflammatory cascades, and an elevated release of neurotrophic agents (such as neuronal growth factors) into the surrounding tissues, which stimulate wound repair and debris clearance to restore tissue homeostasis after a pro-inflammatory response (Cherry et al., 2014). However, in scenarios of pathological neurodegeneration, microglia maintains a persistent activated state, with an uncontrolled release of pro-inflammatory mediators, sustaining a chronic inflammatory state, which is highly injurious to the CNS (Cherry et al., 2014). Furthermore, in neurodegenerative conditions, there is an elevated production of ROS that affects microglial polarization balance, resulting in an increased subpopulation of M1 cells. Activated microglia also release $\cdot O_2^-$ and H_2O_2 that impact on the surrounding neurons, leading to a vicious cycle of microglial activation and neurodegeneration (Rojo et al., 2014).

Recent studies provide evidence that consumption of (poly) phenols-rich diets, as found in fruits, may lower the risk of developing neurodegenerative diseases due to their anti-inflammatory properties (Spencer, Vafeiadou, Williams, & Vauzour, 2012). Indeed, diet may affect human health considering that it can have detrimental effects, or in contrast, being able to attenuate inflammation (Wu & Schauss, 2012). (Poly)phenols, such as flavonoids and phenolic acids, have shown to play important roles in the regulation of inflammatory processes associated with several diseases, including neurodegenerative disorders (Spencer et al., 2012). Raspberries are fruits rich in these bioactive compounds (Bobinaitė, Viškelis, & Venskutonis, 2012) and some studies with raspberry extracts have shown anti-inflammatory capacity (Jo et al., 2015), and neuroprotective effects (Chen, Su, Huang, Feng, & Nie, 2012). However, it remains unclear as to how these compounds exert beneficial effects, what concentrations are necessary, and which are the biologically active forms. In addition, when studying the potential effects of (poly)phenol-rich foods in human health, it is essential to bear in mind that the bioavailability of (poly)phenols is dependent on the modifications that ingested food suffers along the gastrointestinal (GI) tract (Manach, Williamson, Morand, Scalbert, & Remesy, 2005).

As far as we know, this study was the first aiming to assess both the neuroprotective potential and the anti-inflammatory properties of digested extracts from the red raspberry (*Rubus idaeus*). Raspberry extracts were digested *in vitro* to mimic the chemical changes occurring during human digestion and were used in concentrations relevant to circulating metabolites *in vivo*. As a model to access neuroprotective activities of the red raspberry metabolites presented in the GI bioaccessible (GIB) fraction, we used the SK-N-MC human neuronal cell line insulted with hydrogen peroxide (H_2O_2) to induce oxidative stress, a common model used to assess the effects produced by such insult in neuronal cells (Tavares et al., 2012). To evaluate the ability of raspberry digested metabolites to attenuate inflammation we used a N9 murine microglial cell line treated either with H_2O_2 as an oxidant stimulus, or lipopolysaccharide (LPS) as an inflammatory insult (Cui et al., 2002). Remarkably, the digested raspberry metabolites used at physiological levels exhibited anti-inflammatory activity not only by the reduction of Iba1 expression, a microglia activation marker (Ito et al., 1998), but also by inhibiting the release of nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α), two specific markers of the microglial classical pro-inflammatory activation (M1) (Orihuela et al., 2015).

2. Material and methods

2.1. Plant material

Raspberry (*Rubus idaeus*) cv. Himbo-Top was grown in Fataca experimental field (Odemira, Portugal). Berries were harvested at full ripeness (pH 3.00 ± 0.01, soluble solid content 9.0 ± 0.0 (°Brix), titratable acidity 13.3 g (tartaric acid)/L), frozen and then freeze-dried. Fruit extracts were prepared using a hydroethanolic solution (ethanol 50% (v/v)) as previously described (Tavares et al., 2012), prior to *in vitro* digestion.

2.2. In vitro digestion

Phytochemical alterations during digestion were mimicked using the *in vitro* digestion model as published (Tavares et al., 2012). Briefly, the raspberry extract was submitted to conditions that mimic gastric digestion; pH adjusted to 1.7, addition of pepsin and incubation at 37 °C with shaking at 100 rpm for 2 h. Subsequently, small intestine conditions were mimicked by the addition of pancreatin and bile salts, followed by dialysis with a cellulose tube containing $NaHCO_3$ to slowly raise the pH to that of the small intestine. After 2 h incubation at 37 °C, the solution inside the dialysis tubing was collected. (Poly)phenols of original extract and for this fraction were separated using the C18 solid phase and the recovered compounds were then dried in a Speed-Vac Concentrator to suitable phenolic concentrations. The digested fraction after SPE is designated as the gastrointestinal bio-accessible "GIB" fraction.

2.3. Chemical profiling of the extract

2.3.1. Total phenolic measurement

Determination of total phenolic content of both the original and the GIB fraction was performed by the Folin-Ciocalteau method as adapted to a microplate reader (Tavares et al., 2012). Gallic acid was used as a standard and the results were expressed in mg of gallic acid equivalents (mg GAE).

2.3.2. HPLC-MS phenolic profile determination

Digested and original raspberry extracts were dried by rotary evaporation, re-suspended in 5% (v/v) acetonitrile in water and analyzed on a LCQ-DECA system controlled by the XCALIBUR software (2.0, ThermoFinnigan), as reported previously (Tavares et al., 2013). The samples were applied to a C18 column (Synergi Hydro C18 column with polar end capping, 2.0 mm × 150 mm, Phenomonex Ltd.) and eluted over a gradient of 95:5 solvent A:B at time = 0 min to 60:40 A:B at time = 30 min at a flow rate of 200 μL/min. Solvent A was 0.1% (v/v) formic acid in ultra-pure and solvent B 0.1% (v/v) formic acid in acetonitrile. The LCQ-Deca LC–MS was fitted with an ESI (electrospray ionization) interface and analyzed the samples in positive and negative-ion mode. Before the analysis, the system was tuned by using known concentrations of cyanidin-3-glucoside (positive mode) and quercetin-3-glucoside (negative mode) in ultrapure water. Recovery of components was assessed using peak areas for specific *m/z* values generated from the resident software.

2.4. Cell culture and treatments

2.4.1. Neurodegeneration model

Human neuroepithelioma SK-N-MC cells were obtained from the European Collection of Cell Cultures (ECACC) and cultured in Eagle's minimal essential medium (EMEM) (Sigma) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS, Gibco®),