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Oxidative stress response in canine *in vitro* liver, kidney and intestinal models with seven potential dietary ingredients

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HIGHLIGHTS

- The development of canine *in vitro* culture systems with multiple organ-derived cells.
- An assessment of cellular oxidative stress and antioxidant responses by potential food ingredients in canine liver, kidney, and intestinal cells.
- Chemical- and organ-specific oxidative stress and antioxidant properties may implicate a mechanism of toxicity.

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ABSTRACT

In vitro cell culture systems are a useful tool to rapidly assess the potential safety or toxicity of chemical constituents of food. Here, we investigated oxidative stress and organ-specific antioxidant responses by 7 potential dietary ingredients using canine *in vitro* culture of hepatocytes, proximal tubule cells (CPTC), bone marrow-derived mesenchymal stem cells (BMSC) and enterocyte-like cells (ELC). Cellular production of free radical species by denatonium benzoate (DB), epigallocatechin gallate (EPI), eucalyptol (EUC), green tea catechin extract (GTE) and sodium copper chlorophyllin (SCC), tetrahydroisohumulone (TRA) as well as xylitol (XYL) were continuously measured for reactive oxygen/nitrogen species (ROS/RNS) and superoxide (SO) for up to 24 h. DB and TRA showed strong prooxidant activities in hepatocytes and to a lesser degree in ELC. DB was a weak prooxidant in BMSC. In contrast DB and TRA were antioxidants in CPTC. EPI was prooxidant in hepatocytes and BMSC but showed prooxidant and antioxidant activity in CPTC. SCC in hepatocytes (12.5 mg/mL) and CPTC (0.78 mg/mL) showed strong prooxidant and antioxidant activity in a concentration-dependent manner. GTE was effective antioxidant only in ELC. EUC and XYL did not induce ROS/RNS in all 4 cell types. SO production by EPI and TRA increased in hepatocytes but decreased by SCC in hepatocytes and ELC. These results suggest that organ-specific responses to oxidative stress by these potential prooxidant compounds may implicate a mechanism of their toxicities.

1. Introduction

Cellular reactive oxygen species (ROS) are generated endogenously as in the process of mitochondrial oxidative phosphorylation or arise from interactions with chemical stress or radiation (Ray et al., 2012; Yazdani et al., 2014). Endogenous production of ROS at low concentrations is essential for the physiological health of organisms in a normal cellular environment (Yazdani et al., 2014). Oxidative stress occurs with an increase in cellular ROS levels and the inhibition of cellular antioxidant capacity, resulting in direct or indirect ROS-mediated damage of nucleic acids, proteins and lipids. Oxidative stress has been implicated in

Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; CPTC, canine proximal tubule cells; DB, denatonium benzoate; ELC, enterocyte-like cells; EPI, epigallocatechin gallate; EUC, eucalyptol; GTE, green tea catechin extract; RNS, reactive nitrogen species; ROS, reactive oxygen species; SCC, sodium copper chlorophyllin; SO, superoxide; TRA, tetrahydroisohumulone; XYL, xylitol.

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carcinogenesis (Trachootham et al., 2009), neurodegenerative diseases (Shukla et al., 2011), atherosclerosis (Schiffrin et al., 2007), diabetes (Paravicini and Touyz, 2006) and aging (Haigis and Yankner, 2010). Cytotoxic cellular responses derived from ROS have been reported in both *in vitro* human and animal cell culture models (Acevedo-Morantes et al., 2012; Clerch et al., 1996; Garza et al., 2008; Srivastava and Chan, 2007). Ray et al. (2012) reported that ROS regulated several signaling pathways through an interaction with critical signaling molecules, affecting a wide range of cellular processes including proliferation, metabolism, survival and differentiation. ROS, as well as reactive nitrogen species (RNS), have been proposed as an early event linking parent drug or the bioactivation of the drug to hepatotoxicity, and as a direct mechanistic indicator of a compound's hepatotoxic potential (Shuhendler et al., 2014).

Dietary ingredients include vitamins, minerals, herbs or other botanicals, amino acids and other substances or their constituents. The ingredients in pet food are sources of nutrients, flavoring, preservatives and manufacturing processing aids (FDA, 2015). An interest in use of herbs, botanicals and botanical-derived products as food ingredients is rapidly increasing worldwide for the purported benefits of attenuating medical problems and improving overall health status (Abdel-Rahman et al., 2011; Poppenga, 2002). A safety assessment of these ingredients is often limited to the documented history of safe food use without any additional animal toxicity or human clinical data required (Abdel-Rahman et al., 2011). Thus, it would appear that safety screening methods could be improved by collecting data on characterization of chemical- and species-specific toxicity. This is especially crucial when an ingredient with a clean safety record in humans is now added to the food of a different species such as a dog. It is important to consider organ-specific toxicity, including the major metabolic organ the liver, and to the lesser degree the small intestine, or an excretory organ such as the kidney for systemically absorbed food ingredients. In this study, candidate compounds were selected for screening if they meet the following criteria (1) compounds are generally recognized as safe (GRAS) for specific uses; (2) compounds previously reported pet animal toxicity, and/or (3) compounds were subject to the research and development activities of Mars Incorporated. A total of 6 botanical-derived products were chosen to assess their safety/toxicity in dogs. The green tea catechin extracts (GTE), (−) epigallocatechin-3-gallate (EPI) (Mahinka, 2007), eucalyptol (EUC) (European Commission, 2002) and tetrahydroisohumulone (TRA) (FDA, 2001) are affirmed as generally recognized as safe (GRAS) for human consumption. Xylitol (XYL) (Cho, 2013) and chlorophyll-derived sodium copper chlorophyllin (SCC) are FDA-approved food additives (FDA, 2002). Denatonium benzoate (DB), an aversive agent preventing non-drug poisoning in human and animals (US Consumer Product Safety Commission, 1992), was also used for the assessment of their safety/toxicity in dogs.

Several studies reported that the botanical-derived compounds showed concentration- and extraction method-dependent toxicities in humans and/or animals *in vivo* and *in vitro*. Dried green tea derived from *Camelis sinensis* consists of 30% polyphenols called catechins (Graham, 1992). Its major catechin, EPI accounts for 50–80% representing 200–300 mg in a brewed cup of green tea (Khan et al., 2006). Catechins have been attributed with different health benefits including prevention or control of cancers, diabetes, kidney and liver injury, vascular diseases and obesity (Crespy and Williamson, 2004; Thomson et al., 2012), oxidative stress (Lambert et al., 2010; Nasri et al., 2014; Yokozawa et al., 2012) as well as possessing antibacterial and antiviral activity (Inacio et al., 2013; Stoicov et al., 2009). Human hepatic failure associated with hydroalcoholic green tea extracts has been reported in Europe (Bjornsson and Olsson, 2007; Javaid and Bonkovsky, 2006). A high concentration of green tea extract showed histopathological changes in the liver and caused mortality in beagle dogs (Kapetanovic et al., 2009), male Swiss Webster mice (Goodin et al., 2006) and CF-1 mice (Lambert et al., 2010).

DB, also known as lidocaine benzyl benzoate, is a bitter substance used as a repellent to prevent animals from feeding on trees and other woody plants. The exposure level of DB in repellent is expected to have negligible adverse effects to mammals (The Health Canada Pest Management Regulatory Agency, 2012). In animal studies DB showed acute oral LD_{50} 612 mg/kg in rats; for mice 865 mg/kg; for guinea pigs 805 mg/kg (US Consumer Product Safety Commission, 1992). Hansen et al. (1993) reported that dogs are much less sensitive to aversive bittering agent DB. Miyata et al. (2014) reported that DB aided digestion through the increase in cholecystokinin release in mouse intestinal enteroendocrine cell line, STC-1. DB is a ligand for the bitter taste receptor (T2R), resulting in an increase in antimicrobial peptide secretion in primary human sinonasal epithelial cells (Lee et al., 2014). The monoterpene oxide 1, 8-cineol, also known as eucalyptol (EUC), is a major constituent of eucalyptus oil, isolated from *Eucalyptus globulus* (Dörsam et al., 2014), and to a lesser degree, from herbs like *Rosemarinus officinalis* (Kovar et al., 1987). EUC has antiparasitic activity against the *Leishmania* species (Machado et al., 2014), antiviral activity against the herpes simplex virus (Schnitzler et al., 2001), as well as analgesic and anti-inflammatory activity (Takaishi et al., 2012). EUC caused a significant concentration-dependent increase in oxidative DNA damage without cytotoxicity in human colon cancer cells (Dörsam et al., 2014). A dose of 381 mg/kg of EUC caused the accumulation of eosinophilic protein droplets in rat kidney proximal tubule cells (De Vincenzi et al., 2002). Accidental oral ingestion of EUC in a 3-year-old boy caused nervous system depression (Patel and Wiggins, 1980).

Humulus lupulus L. (Cannabaceae), commonly known as hops, has been used as a preservative and bittering ingredient in the brewing industry and also for medicinal purposes (Zanoli et al., 2005). Hops α-acids (humulones) and β-acids (lupulones) are constituents of the essential bitter resin. During wort-boiling in the brewing process, hydrophobic humulones are isomerized to isohumulone and further hydrogenation yields tetrahydroisohumulone (TRA) (Chappel et al., 1998; Barth, 2013). A dose of 50 mg/kg of TRA caused an increase in serum alkaline phosphatase levels in dogs that did not affect liver health with this dose being set as the no observed adverse effect level (NOAEL) (Chappel et al., 1998). Humulones can suppress the induction of tumor necrosis factor α (TNFα)-induced cyclooxygenase-2 (COX-2) expression in murine osteoblasts MC3T3-E1 by blocking NF-kB and NF-IL6 (Yamamoto et al., 2000).

Sodium copper chlorophyllin (SCC) is a water-soluble sodium copper salt from the natural green pigment chlorophyll, widely used as a food dye (Domijan et al., 2015; Tumolo and Lanfer-Marquez, 2012). SCC has antimutagenic and anticarcinogenic activity by binding to DNA intercalating compounds, potentially preventing chemical-induced mutagenesis and carcinogenesis (Domijan et al., 2015; Osowski et al., 2010; Tumolo and LanferMarquez, 2012). SCC also caused a reduction in ROS-induced DNA strand breakage and 8-hydroxy deoxyguanosine (8-OH-dG), a marker of oxidative stress (Park et al., 2003). El-Ghor et al. (2014) reported that chlorophyllin caused a dose-dependent reduction in titanium dioxide (TiO_2) nanoparticle-induced micronuclei and DNA damage, p53 mutation and hepatic malondialdehyde levels and improved antioxidant activity by induction of superoxide dismutase, catalase and glutathione peroxidase activity and glutathione levels.

Xylitol (XYL) is a 5-carbon sugar alcohol, used as a substitutive sweetener for diabetic patients and is added to chewing gum to prevent dental caries (Riley et al., 2015; Xia et al., 2009). Xylitol at 1