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In vitro safety assessment of food ingredients in canine renal proximal tubule cells

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ABSTRACT

In vitro models are useful tools to initially assess the toxicological safety hazards of food ingredients. Toxicities of cinnamaldehyde (CINA), cinnamon bark oil, lemongrass oil (LGO), thymol, thyme oil (TO), clove leaf oil, eugenol, ginger root extract (GRE), citric acid, guanosine monophosphate, inosine monophosphate and sorbose (SORB) were assessed in canine renal proximal tubule cells (CPTC) using viability assay and renal injury markers. At LC_{50}, CINA was the most toxic (0.012 mg/ml), while SORB the least toxic (>100 mg/ml). Toxicities (LC_{50}) of positive controls were as follows: 4-aminophenol (0.15 mg/ml in CPTC and 0.083 mg/ml in human PTC), neomycin (28.6 mg/ml in CPTC and 27.1 mg/ml in human PTC). XYL displayed lowest cytotoxic potency (LC_{50} = 82.7 mg/ml in CPTC). *In vivo* renal injury markers in CPTC were not significantly different from controls. The LGO toxicity mechanism was analyzed using qPCR and electron microscopy. Out of 370 genes, 57 genes (15.4%) were significantly up (34, 9.1%) or down (23, 6.2%) regulated, with the most upregulated gene *gsta3* (~200-fold) and the most affected pathway being oxidative stress. LGO induced damage of mitochondria, phospholipid accumulation and lack of a brush border. Viability assays along with mechanistic studies in the CPTC model may serve as a valuable *in vitro* toxicity screening tool.

1. Introduction

Many chemical ingredients are commonly used in human cosmetics, food, dentistry and alternative medicine for their purported beneficial activities. However, the potential toxicity for many of these components in veterinary species such as the dog is unknown. A recent National Research Council study on the safety of dietary supplements in companion animals suggested that *in vitro* data in target animal species be collected to begin to serve as a bridge with existing data in human and laboratory rodents (NRC, 2009).

Such compounds include eugenol (EUG) (major component of clove leaf oil) with its anti-oxidant activity (Ogata et al., 2000) and clove leaf oil (CLO) having anti-inflammatory, anti-oxidant and anti-microbial properties (Chaieb et al., 2007). Lemongrass oil (LGO) is purported beneficial due to its broad spectrum of attributes such as anti-oxidant, bactericidal, fungicidal, anti-inflammatory, anti-cancer and anti-leishmanial properties (Sacchetti et al., 2005; Silva et al., 2008; Bachiega and Sforcin, 2011; Sharma et al., 2009; Santin et al., 2009) and ginger root extract (GRE) having anti-oxidant (Sacchetti et al., 2005) and anti-cancerous properties (Shukla and Singh, 2007). In addition, cinnamon bark oil (CBO) has anti-microbial and anti-carcinogenic properties, and has the potential to be used in natural remedies (Unlu et al., 2010); cinnamaldehyde (CINA), a major constituent of CBO (~70%) is an active anti-fungal agent (Singh et al., 1995); thyme oil (TO) has anti-tumoric effects in *in vivo* and *in vitro* studies (Ait M'Barek et al., 2007), while thymol (THYM), an abundant constituent of TO (~40%) has been described to have anti-oxidant and bactericidal properties (Yanishlieva et al., 1999; Bagamboula et al., 2004). Guanosine monophosphate (GMP), inosine monophosphate (IMP), xylitol (XYL) and sorbose (SORB) are active as taste potentiators and food additives as alternative substitutes for glucose-based sweeteners, respectively (Maga and Yamaguchi, 1983; Gare, 2003; Hyvonen et al., 1982).

Cytotoxicity of EUG, widely used in dentistry is likely mediated via ROS-independent mechanisms, possibly involving the production of phenoxyl radicals in submandibular cell lines (Atsumi et al., 2005), GSH depletion in human osteoblasts (Ho et al., 2006), while hepatotoxic effects are primarily mediated through its quinone methide metabolite (Thompson et al., 1998). CLO

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toxicity has been attributed to EUG constituting ~80% of the oil. Morphological changes include membrane damage resulting in necrosis or apoptotic cell death (Prashar et al., 2006), mitochondrial damage (Park et al., 2005) and membrane ion efflux (Thompson et al., 1995). *In vivo* CLO studies in rats reported moderate alterations in the kidney such as desquamation of the tubular epithelial cells (Shalaby et al., 2011).

Citral, a major component of LGO inhibits nitric oxide production (Lee et al., 2008) and induces phase II drug metabolizing enzyme activity (Nakamura et al., 2003). *In vitro* toxicity studies with LGO and lemon myrtle oil in various mammalian cell lines were comparable (Dijoux et al., 2006; Manosroi et al., 2006; Hayes and Markovic, 2002). *In vitro* toxicity of essential oils is generally caused by membrane damage and induction of apoptosis and necrosis (Bakkali et al., 2008). However, some essential oils, including LGO may be phototoxic (Dijoux et al., 2006) and might penetrate the cell without causing cell membrane, protein or DNA damage (Bakkali et al., 2008).

GRE is widely used as a food additive with anti-oxidant activity comparable with butylated hydroxytoluene (BHT), indicating its potential as a natural preservative (Stoilova et al., 2007). Hepatotoxic effects include superoxide generation, reduction of nitric levels and GSH depletion (Vijaya Padma et al., 2007).

Citric acid (CA) is used in the food industry as an anti-oxidant as well as in human and veterinary periodontal procedures as a root canal irrigant, which enlarges the canal and increases the permeability of dentine (Ririe et al., 1980). Since periodontal tissue might be altered by such an irrigant, its cytotoxicity has been studied in several cell lines (Zaccaro Scelza et al., 2001; Amaral et al., 2007). GMP and IMP are naturally occurring 5'ribonucleotides used in the food industry as flavor enhancers (Maga and Yamaguchi, 1983).

SORB, a monosaccharide of the keto-hexose group, is a potential additive in human foods (Hyvonen et al., 1982). Although not reported to be toxic to humans, it may cause hemolysis of canine red blood cells *in vitro* (Keller and Kistler, 1977).

The kidney consists of diverse cell types with the proximal tubule cells (PTC) being a primary target cell for toxicant exposure since it is the site of active transport, excretion of xenobiotics, reabsorption of secreted solutes and most importantly retention of an absorbed fraction of certain nephrotoxicants such as aminoglycosides (Riviere, 1985). Therefore, we have focused on isolation of the PTC in the canine species to evaluate potential toxicants and dietary supplements in dogs. In order to better interpret the toxicity of the selected chemicals in pet food ingredients, the known nephrotoxicants 4-aminophenol (4-AP) and neomycin (NEO), and XYL known to be hepatotoxic to dogs served as controls in the viability assays. In PTC, toxicity of 4-AP is exerted via enzymatic and nonenzymatic oxidation (Hallman et al., 2000). Aminoglycoside antibiotics, such as NEO are widely known classic nephrotoxicants *in vivo* (Riviere, 1985). Signs of aminoglycoside toxicity include changes in the renal cortex including accumulation of phospholipids in lysosomes, inhibition of lysosomal enzymes, decreased reabsorption and shedding of brush-border enzymes (Watanabe, 1978). XYL is a common food ingredient categorized as a sugar alcohol present in small amounts in vegetables and fruits. It is used as a substitute for sucrose and despite its anti-cariogenic properties in humans (Gare, 2003) it causes hypoglycemia (Piscitelli et al., 2009) and potentially life-threatening hepatotoxicity in dogs (Dunayer and Gwaltney-Brant, 2006). Studying the potential toxicity of novel drugs/food ingredients in the PTC model, demands biomarkers' standards such as sensitivity, specificity and reliability to screen for early diagnosis and prognosis of acute renal injury. Several biomarkers (e.g. cystatin C, KIM-1, β_2-microglobulin, albumin, total protein, TFF3, RPA-1, clusterin) can capture early kidney injury *in vivo* (Bonventre et al., 2010). In addition to canine cell culture lines, we utilized human proximal tubule cells (HPTC) to correlate to CPTC data using biomarker endpoints of cell death and profiles of the kidney injury markers (KIM-1, clusterin, cystatin C) after exposure to the aforementioned ingredients.

The objective of this study was to begin a hazard assessment of the cytotoxicity of thirteen common food ingredients and control substances (CBO, CINA, TO, THYM, CLO, EUG, LGO, GRE, SORB, GMP, GMP + IMP, CA and XYL) in CPTC and to further characterize the mechanism of LGO toxicity, since LGO toxicity was significantly higher in CPTC than other canine cell lines. There is minimal information about the molecular mechanism of essential oil toxicity. Therefore, we screened for multiple metabolic pathways using a high-throughput genomic array to elucidate the relationships among genes of the affected pathways and to gain insight into mechanism of LGO toxicity.

2. Materials and methods

2.1. Chemicals

EUG, XYL, SORB, GMP, IMP, CBO, CINA, TO, THYM, CA, 4-AP and NEO were purchased from Sigma–Aldrich (St. Louis, MO), while CLO, LGO and GRE were purchased from Spectrum Chemicals (New Brunswick, NJ).

2.2. Animals

Dogs used in this study were mixed breeds ranging in ages from 1.5 to 9 years ($n = 3$ for ingredient testing; $n = 3$ for nephrotoxicant testing; $n = 4$ for qPCR array). Dogs were housed individually and provided with food and water *ad libitum* and housed under normal environmental conditions. Prior to euthanasia, dogs were first anesthetized with 1:1 mixture of ketamine and xylazine (i.v., 55–74 μl/kg) and within ten min of anesthesia were euthanized with sodium pentobarbital (i.v., 222 μl/kg) and the kidneys were removed immediately. Kidneys for these studies were obtained from dogs euthanized by the animal shelter for clinical issues after owner consent. The use of the tissue for research purposes was approved by the university's Institutional Animal Care and Use Committee protocol 3214.

2.3. Kidney collection and renal cell isolation

PTC isolation was adapted from Vinay et al. (1981) and then modified for CPTC. Fresh dissected kidneys were perfused with 50 ml of cold heparin in Hank's balanced salt solution and transported on ice-cold saline. Upon arrival, kidney cortex was removed, sliced and weighed. Ten-gram pools of the cortex slices were chopped, ground with a mortar and pestle, washed in carbonated/oxygenated Krebs Henseleit saline (KHS) buffer and treated with collagenase type I (Sigma–Aldrich, St. Louis, MO)/KHS solution for 1 h at 37 °C. Then, the cell suspension was mixed with ice-cold buffer, strained through cheese cloth and a 100 μm cell strainer. Cells were washed with KHS buffer (47 g, 5 min) and separated in 50% Percoll (Sigma–Aldrich, St. Louis, MO) by density gradient centrifugation at 23,000*g*, 30 min, 4 °C, which rendered four distinctive bands. Each of the cell layers were collected, washed and examined microscopically. Viability was 20%, and total live cells ranged from 1 to 10 million cells depending on the size of the kidneys, age and health. The lowest Percoll band, consisting mainly of tubular fragments was collected and washed in KHS and supplemented with culture medium EpiCM (ScienCell, Carlsbad, CA) and plated on poly-L-lysine-coated T75 flasks and incubated at 37 °C (5% CO_2). Once confluent, trypsinized cells were cryopreserved in EpiCM containing 20% FBS and 10% DMSO.