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Effects of a vinegar-based multi-micronutrient supplement in rats: A multi-pronged assessment of dietary impact

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ABSTRACT

We determined the effects of continuous access to drinking water with a vinegar-based multi-micronutrient (VMm) supplement containing rice and fruit vinegars, vitamins, organic acids and sugars during gestation, lactation, and early adulthood in rats. Pregnant rats were provided with reverse-osmosis water or VMm water from the start of pregnancy through the time of weaning. Weaned pups consumed the same drinking water for 3 to 12 additional weeks. We examined fecal metabolite and microbial profiles, and other physiological parameters. Body weights were less in rats that drank VMm water. Thirty fecal metabolites involved in amino acid and dipeptide metabolism were significantly altered in VMm-supplemented rats. Analysis of microbial 16S rRNA showed enrichment of bacteria in the family S24-7 in VMm-supplemented rats, and one in Ruminococcaceae in controls. Our data show that a VMm-containing beverage can alter growth, and gut metabolism and microbial community. Future work to correlate these parameters is warranted.

1. Introduction

A dietary supplement is a product that (a) is meant to augment the diet, and (b) contains vitamins, minerals, amino acids, botanical extracts or other similar ingredients (FDA, 1994). Common among supplements are micronutrients and vitamins, since certain dietary patterns may not provide all the optimal nutrients from food and water intake alone. Vinegars have been ingested for millennia and are an important element in Asian, European, Western and other traditional cuisines. Vinegar has been used for preservation of various foods and is often used for flavoring and pickling.

Recent research has indicated that vinegar affects glucose metabolism and alters lipid profiles in rats and humans (Hlebowicz, Darwiche, Bjorgell, & Almer, 2007; Johnston, Steplewska, Long, Harris, & Ryals, 2010; Naziroglu et al., 2014; Petsiou, Mitrou, Raptis, & Dimitriadis, 2014). Vinegar has also been shown to attenuate experimentally induced colitis in mice, via suppression of inflammation (Nishidai et al., 2000; Shen et al., 2016; Shimoji et al., 2002). Fruit vinegars have been reported to improve immune function (Bounihi et al., 2014; Cha, Moon, Soh, Oh, & Choi, 2006; Lee, Kim, Do, Kim, & Kwon, 2014). Many of these potential benefits from consumption of vinegars in animals and humans might be explained by their ability to alter the gut microbiome and the metabolite profile. Increasing evidence suggests that alterations in gastrointestinal flora and metabolites can affect health via systemic processes (Arpaia et al., 2013; Chen et al., 2014; Huang et al., 2013). Pairogen® (Akatsuka Co., Tsu, Japan) is a beverage that contains ferrous-ferric chloride (FFC®) water, rice and fruit vinegars (e.g., apple, Japanese apricot and persimmon), sugars, citric and malic acid and vitamins B and C (Hirobe, 2009). The rice and fruit vinegars are obtained by fermentation of rice and fruit sugars, respectively. Adding Pairogen to drinking water has been reported to improve mouse survival when challenged with intravenous administration of *Rhodococcus* (Yimin et al., 2012). Pairogen-supplemented mice cleared bacteria from liver and spleen significantly faster. These effects were passed on to their F1 progeny. Enhanced IL-10 and heme oxygenase-1, decreased TNF-α and IL-6 expression in *Rhodococcus aurantiacus*-stimulated

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peritoneal macrophages were also shown in F1 mice consuming Pairogen (Yimin et al., 2012).

Although the effects of Pairogen have been studied in a disease model, as described above (Yimin et al., 2012), no data on the effects of chronic consumption of this drink in healthy animals are available. The objective of this study was to determine the effects of continuous access to a vinegar-based multi-micronutrient supplement (Pairogen) in drinking water during gestation, nursing (lactation) and early adulthood in rats. We examined fecal metabolite and microbial community profiles, as well as selected physiological parameters such as body weight, body fat and bone composition, blood chemistry, and gut absorption of iron, in order to evaluate the effects of ingestion of a fruit vinegar-containing beverage.

2. Materials and methods

2.1. Vinegar-based multi-micronutrient supplement

The multi-micronutrient supplement used in this study was obtained as a concentrated formulation (Pairogen®) from Akatsuka Garden Company (Tsu City, Japan). The Pairogen concentrate contains water, rice and fruit vinegars (e.g., apple, Japanese apricot and persimmon), sugars, citric and malic acid and vitamins B and C. The sample was diluted 1:100 with reverse-osmosis water and filtered through a 0.45 µm pore size membrane and analyzed at Akatsuka Co. (Japan). Organic acids, vitamins, amino acids and sugars were measured using liquid chromatography on an Agilent 1100 Series liquid chromatograph (Agilent Technologies, Santa Clara, CA) with auto sampler. Elemental analysis was performed by inductively coupled optical emission spectroscopy (ICP-OES) (Optima 5300 DV, Perkin Elmer, Billerica, MA). Anions were measured by ion chromatography (ICS-3000, Dionex, Sunnyvale, CA). The column composition, temperature, mobile phase, flow rate and detector varied depending on the type of compounds being analyzed (See Online Supplement, Methods). Additional analyses for small molecules in Pairogen concentrate were performed at Metabolon, Inc. (Durham, NC) using liquid chromatography/mass spectroscopy (LC/MS) or gas chromatography/MS (GC/MS). Details of methods for VMm analyses are available in Online Supplement.

2.2. Experimental design

The animal protocols in this study were approved by the Harvard Medical Area Animal Care and Use Committee. Fig. 1 outlines the overall experimental design. A total of twelve female Sprague-Dawley rats 2 days after conception were obtained from Taconic Farms (Germantown, NY) and housed individually in standard pasteurized polycarbonate microisolator cages under controlled conditions of temperature, humidity, and light at the Harvard Center for Comparative Medicine. They had access to commercial chow (PicoLab Rodent Diet 5053, Framingham, MA) and designated drinking water *ad libitum* throughout pregnancy and lactation.

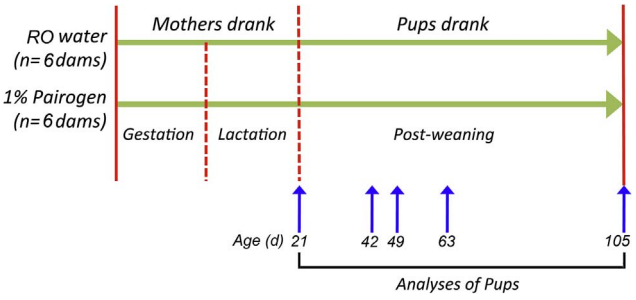

Fig. 1. Experimental design. Pregnant rats were obtained at gestational age E2. Pregnant rats were provided with drinking water supplemented with 1% v/v Pairogen™ (n = 6 rats) or with reverse-osmosis (RO) water (n = 6 rats). The designated drinking water was provided throughout the period of pregnancy and lactation. At age 21 days, the pups were weaned and were then provided the same drinking water assigned previously until euthanasia at times up to age 105 days. Fecal samples were collected at age 21 days and 42 days. Additional rats were tested for gut absorption of iron at age 49 days. Body composition (bone density and body fat) was analyzed in selected rats at age 63 and 105 days.

These pregnant rats were provided with either reverse-osmosis water or with the same reverse-osmosis water with 1% VMm supplement. In a previous pilot experiment, higher VMm concentrations resulted in a decrease in water consumption. The designated drinking water was provided throughout the period of pregnancy (21–22 days). Right after birth, the litter size was randomly culled down to 10 pups/litter each with male to female ratio of 5 to 5. The designated drinking water was continuously provided throughout lactation. As the pups aged, they might have consumed not only their mother's milk but also the drinking water and chow provided to their mothers. At age 21 days, the pups were weaned and were then provided with the same drinking water assigned previously until euthanasia at times up to age 105 days. This protocol provided a long-term consumption of VMm, encompassing gestational, lactational and early developmental stages including the onset of puberty. In an initial experiment, 10 pups from 1 control and 10 pups from 1 VMm dam were used for fecal metabolomics analysis at age 21 and 42 days. Then, in a larger experiment, 10 pups from 5 dams/treatment group (1 male and 1 female per dam) were used for fecal metabolomics and microbiome analyses at age 21 and 42 days. This experimental design explored the importance of greater genetic variability among rats in each treatment group. These same rats were analyzed for hematological parameters (42 and 63 days) and for body composition (bone density and body fat, 63 days). Additional rats were also analyzed for body composition at age 105 days and for gut absorption of iron at age 49 days.

2.3. Assessment of iron absorption in the gastrointestinal tract

We used radioisotope of iron (^{59}Fe) as tracer in a pharmacokinetic study to determine if consumption of VMm affects iron bioavailability from the gastrointestinal tract. We determined if iron absorption in the gut, as well as iron clearance from the blood, and tissue distribution were affected in VMm-exposed pups. $^{59}FeCl_3$ was purchased from Perkin Elmer (Boston, MA) and diluted with 1:50 M excess of ascorbic acid immediately prior to the experiment to reduce $^{3+}$Fe to $^{2+}$Fe. A total of 6 rats from each treatment group were dosed by gavage with ^{59}Fe in this buffer at 1 ml/kg volume dose and equivalent radiation dose of 150 µCi ^{59}Fe/kg. Each rat was anesthetized with up to 4% vaporized isoflurane (Halocarbons Lab, North Augusta, SC) prior to gavage dosing. Blood samples were sequentially obtained from the tail vein over a 72-h period (15, 30, 60, 90, 120, 240, 480 m, 24, 48, and 72 h). Plasma and red blood cells were separated for radioisotope analysis.

Since the blood levels of ^{59}Fe represent the amount absorbed from the gut minus the amount cleared from the circulation, another set of 4 rats/group was intravenously injected with the same dose of ^{59}Fe via the penile vein. Blood samples were similarly obtained from the tail vein. At 72 h post-dosing with $^{59}FeCl_3$, all rats were humanely killed with overdose of isoflurane anesthesia, exsanguinated via the abdominal aorta, and tissue samples collected. The gastrointestinal tract (GIT) was divided into segments after removal of the luminal contents. Radioactivity of samples of tissue, plasma, and red blood cells was measured using a WIZARD 1410 gamma counter (Perkin Elmers, Waltham, MA). Data were analyzed and expressed as tissue concentration (µCi/g) and % of the administered dose.

Pharmacokinetic (PK) analyses were performed on plasma and RBC levels during the first 24 h to compare PK parameters for ^{59}Fe between control and VMm groups using the WinNonlin Software version 5.2 (Pharsight Corp, Mountain View, CA). PK parameters such as half-life (time during which one half of ^{59}Fe dose is cleared from the plasma), total area under the plasma concentration-time curve (AUC) (an estimate of ^{59}Fe in the plasma), total body clearance (CL) (rate of loss of