Impact of Aging on Gastrointestinal Permeability During Hyperthermia

Statistical Analysis Plan

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Study protocol

Participants: Young (18-39 years) and older (≥ 65 years) participants will be recruited from the greater Dallas-Fort Worth metropolitan area. Exclusion criteria will include: 1) known heart disease or other chronic medical conditions currently requiring regular medical therapy such as cancer, diabetes, uncontrolled hypertension, or uncontrolled hypercholesterolemia; 2) currently taking tricyclic antidepressants, loop diuretics, centrally acting calcium channel blockers, or beta-blockers; 3) abnormalities detected suggestive of provocable ischemia, undetected cardiac disease, or resting left bundle branch block on screening electrocardiogram; 4) current smoker or regularly smoked within the past three years, 5) body mass index greater than or equal to 30 kg/m^2; 6) known gastrointestinal issues such as Crohn's disease or ulcerative colitis, and 7) pregnant (confirmed in young females using a urine pregnancy test).

Study design and experimental controls: Enrolled participants will complete a thermoneutral control visit and a hyperthermia trial. Participants will adhere to the following pre-test guidelines: not use non-steroidal anti-inflammatory drugs for at least 1 week, antibiotics for at least 3 weeks, and steroids for at least 6 weeks prior to enrollment and regular/ongoing use of supplements known to influence the intestinal barrier (i.e., probiotics, glutamine, bovine colostrum, etc.). All testing will take place between 8:00 and 9:00 AM after an overnight fast. Additionally, participants will be asked to refrain from aerobic/resistance exercise for 24 hours, alcohol for 24 hours, and caffeine for 8 hours prior to testing.

Control visit: Participants will ingest the multi sugar drink (described in detail below) dissolved in 100 mL of water for determination of resting (baseline) intestinal permeability. They will be asked to collect their urine for 3 hours into 3-L opaque polypropylene containers. During this time participants will remain rested (seated with minimal movement). Collected urine samples were then measured for volume and mixed well before two 1.5 mL aliquots will be taken and stored at −80°C.

Hyperthermia trial: Upon arrival, participants will confirm adherence to the pre-test guidelines and voided their bladder into a polypropylene container to confirm euhydration and to calculate their baseline urine flow rate. Two 1.5 mL aliquots will be taken and centrifuged at 20,800 x g for 15 minutes in 4°C. 1 mL of each supernatant will be stored

at −80°C. Before instrumentation, participants' body mass was measured using a precision balance scale with ± 10 g accuracy (Mettler Toledo, OH). Core temperature will be measured using an orally ingestible telemetric pill (e-Celsius performance pill ®, BodyCap©, Caen, France) taken no less than 1 hour before the baseline period. Mean skin temperature will be obtained as the weighted average of local temperatures measured via thermocouples attached to the skin surface on the chest (30%), arm (30%), anterior thigh (20%), and calf (20%). Heart rate will be obtained from an electrocardiogram (GE Medical Systems, Madison, WI). Blood pressure will be measured by using an arm cuff with a microphone placed over the brachial artery to detect Korotkoff sounds triggered from the ECG signal (Tango M2 Stress Test Monitor, SunTech Medical). Following instrumentation, participants will don a tube-lined suit that covered the entire body except for the head, hands, and feet. To manipulate core and skin temperatures participants will exercise on a cycle ergometer (Lode Corival Recumbent, Netherlands) at 20 watts while the temperature of the water circulating through the tube-lined suit will be increased and maintained at 50°C. When core temperature is increased by 0.5°C above baseline, participants will ingest the multi sugar drink dissolved in 100 mL of water, heated to core temperature, for assessment of intestinal permeability (described in detail below). Participants will be heated to thermal tolerance which was defined as: 1) a core temperature increase of 2°C, or 2) the time at which the participant expressed that they were unable to continue or had sustained symptoms of presyncope. After thermal tolerance is reached, the temperature of the water perfusing the tube-lined suit will be reduced to cool the participant. After a brief period of cooling, participants will towel off and measure their nude body mass. Participants will then drink at least 500 ml of water but remain rested and fasted for the remainder of the trial. Any urine produced for the 3 hours following ingestion of the multi sugar drink will be collected into 3-L opaque polypropylene containers. Collected urine samples will be measured for volume and mixed well before two 1.5 mL aliquots are taken and centrifuged at 20,800 x g for 15 minutes in 4°C. 1 mL of each supernatant will be stored at −80°C.

Blood sampling and analysis: Blood samples will be collected through venipuncture of an arm vein into heparin, ethylenediaminetetraacetic acid (EDTA), or serum separator Vacutainers® in the seated upright position prior to heating (baseline) and at the point of

thermal tolerance (end). We will measure hematocrit (microcapillary technique) and hemoglobin (ABL90 Flex, Radiometer, Brønshøj, Denmark) to calculate changes in plasma volume (Dill & Costill, 1974). Blood samples will be centrifuged to isolate plasma/serum and aliquots stored at -80°C for later analyses. We will measure plasma (heparin) intestinal fatty acid binding protein (I-FABP), plasma (EDTA) lipopolysaccharide binding protein (LBP), and plasma (EDTA) soluble cluster of differentiation 14 (sCD14) using enzyme-linked immunosorbent assays. In addition, to assess changes in renal function we will measure plasma (heparin) creatinine (Medica RA chemistry analyzer) and cystatin C (Tosoh AIA-360). Stored serum samples will be assayed using a chemo/cytokine multiplex assay (LHC0009M ThermoFisher Scientific, Waltham, MA) run on a Luminex™ MAGPIX platform (xMAP Technology, San Diego, CA).

Urine analysis: Stored urine samples will be assayed for tissue inhibitor of metalloproteinase 2 (TIMP-2) insulin like growth factor binding protein 7 (IGFBP7), neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule-1 (KIM-1) via enzyme-linked immunosorbent assays (RayBiotech Life, Peachtree Corners, GA). Urinary biomarkers will be normalized to urine flow rate.

Multi sugar drink: Intestinal permeability will be assessed using a multi sugar drink test as described elsewhere (van Wijck *et al.*, 2013). The drink will be modified to include only 1 g of lactulose (Kristalose®, Cumberland Pharmaceuticals, Nashville, TN), 1 g of sucrose (107653, Sigma-Aldrich, St. Louis, MO), and 0.5 g of L-rhamnose (W373011, Sigma-Aldrich, St. Louis, MO). The urinary recovery of each ingested sugar (lactulose, sucrose and rhamnose) will be determined by multiplying the measured concentration of each sugar by the total volume of urine collected and dividing by the dose administered. If lactulose or sucrose is not detected in the sample, the L/R ratio or sucrose excretion will be assumed to be 0.