THESIS

ACUTE BEET JUICE INGESTION IMPROVES ESTIMATES OF INSULIN SENSITIVITY IN OBESE ADULTS

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ABSTRACT

ACUTE BEET JUICE INGESTION IMPROVES ESTIMATES OF INSULIN SENSITIVITY IN OBESE ADULTS

Poor glucose regulation is strongly associated with low nitric oxide (NO) bioavailability; a characteristic that may be improved with stimulation of NO generating pathways. For example, endothelial nitric oxide synthase null mice demonstrate improved glucose metabolism following sodium nitrate ingestion. Dietary nitrates are sequentially reduced in the oral and gastric cavities to NO, a process that is attenuated by rinsing with an antibacterial mouthwash. We hypothesized that acute dietary nitrate consumption will improve glucose tolerance. 9 sedentary, healthy, obese adults (2 male; body mass index: 33.7 ± 4.0 kg/m^2: age: 45±7 years; mean ± SE) were studied. Using a randomized crossover design, four oral glucose tolerance tests were performed (equal carbohydrate load). To assess the influence of dietary nitrate, subjects consumed either 500mL of beet juice + 25g glucose, or 500mL of water + 75g glucose, with and without prior antibacterial mouthwash use. Beet juice was selected because it is rich in nitrate. Venous blood samples were collected for the determination of glucose and insulin concentrations. Neither the circulating glucose nor insulin responses were influenced by beet juice and/or mouthwash (P>0.05). However, the Matsuda Index, an estimate of insulin sensitivity, was greater for beet juice compared with beet juice preceded by mouthwash (104.6 ± 11.7 *vs*. 83.5 ± 11.1; P<0.05). These preliminary data suggest that acute dietary nitrate ingestion may promote insulin sensitivity in obese adults.

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CHAPTER I

REVIEW OF LITERATURE

Type 2 Diabetes

25.8 million (8.3%) Americans currently have type 2 diabetes (1); this is roughly double the number of cases in 1970 (2). This trend is expected to continue, leading to 1 in 3 adults diagnosed by 2050 (1,2). In response, numerous treatment options have been developed for those affected. Oral anti-diabetic agents are being prescribed to over half of patients with type 2 diabetes (3). However, lifestyle modification through diet and exercise remains to be a commonly recommended treatment for diabetes (4).

Glucose Homeostasis and the Pancreatic Hormones

Regulation of whole body glucose homeostasis is central to human metabolism and is the fundamental insufficiency in diabetes. The rates of appearance (R_a) and disappearance (R_d) ultimately determine the concentration of glucose in the blood. The glucose R_a is primarily determined by intestinal absorption of glucose and hepatoportal glucose uptake during the postprandial period, whereas in a fasted state, hepatic and renal glucose production and release are the primary determinants. The glucose R_d is controlled by the rate of uptake into tissues; this occurs in insulin-dependent and

independent tissues. During the postprandial period, insulin-dependent glucose disposal is much more influential than during a fast.

The pancreas plays a pivotal role in glucose homeostasis by sensing the plasma glucose concentration and releasing hormones in response. The pancreatic Islets of Langerhans contain specialized cells called α and β-cells that produce glucagon and insulin, respectively. When plasma glucose concentrations fall, such as during a fast, the α cells respond by secreting glucagon. A primary target of glucagon is the hepatocytes in the liver where it stimulates gluconeogenesis and glycogenolysis, which together act to supply glucose for the plasma. When plasma glucose rises such as during the postprandial period, the β-cells secrete insulin. Insulin has numerous actions, but with regard to glucose homeostasis its main actions are at the hepatocyte, adipose tissue and skeletal muscle. In hepatocytes, insulin opposes the actions of glucagon by stimulating glycogenesis and glycolysis. In skeletal muscle and adipose tissue, insulin stimulates glucose uptake and inhibits lipolysis. The absolute magnitude of glucose uptake into the skeletal muscle is larger than other organs due to the skeletal muscle's large mass and its ability to store glucose as glycogen. Although glycogen concentrations are highest in skeletal muscle when expressed as an absolutely quantity, liver glycogen stores are a great deal larger on a per unit mass basis. When these processes are disrupted, homeostasis is lost and hyper- or hypoglycemia result.

Pancreatic Hormones

Insulin Secretion

After a meal, insulin is the most abundant hormone in circulation and facilitates proper storage of the nutrients absorbed from a meal. Insulin is synthesized and released from the β-cells in the Islets of Langerhans of the pancreas in response to elevated glucose (5). Insulin exocytosis is inherently linked to glucose metabolism in the β-cell (5). In the β-cells, glucose absorption occurs by facilitated diffusion, through the insulin-independent glucose transporter, GLUT-2 (6) and is subsequently phosphorylated by glucokinase (7). Glucokinase is considered a "glucose sensing" enzyme and is the rate limiting step in glucose metabolism in pancreatic β cells (8). Glucokinase has a high K_m of ~14.7mM (9), compared with a normal physiologic blood glucose concentration range of 4-7mM (10). This relationship allows β-cell glucose metabolism to increase throughout the physiologic range and is critical to maintaining glucose homeostasis (11) and glucose-stimulated insulin secretion (GSIS) in the β-cells (12,13).

Insulin secretion is pulsatile (14) and biphasic (15). The size of each secretory burst is determined by the magnitude of the hyperglycemia (16). GSIS involves a series of intracellular events that are triggered by a rise in glucose phosphorylation by glucokinase and leads to calcium influx, membrane depolarization and insulin granule exocytosis (17). Membrane depolarization is mediated by adenosine triphosphate (ATP) -sensitive potassium channels (K_{atp}), which close in response to an increase in the ATP:ADP ratio (18). Depolarization opens the L-type Calcium channels, located next to the insulin granules, allowing calcium to enter the cell (19). Mitochondrial

proteins such as the α ketoglutarate carrier (20) and reduced cytochrome c (21) are released into the cytosol during calcium influx and stimulate insulin exocytosis, but the exact mechanism of calcium-induced insulin release remains unclear. We do know, however, that extracellular calcium influx is required for granule exocytosis (22).

Glucagon Secretion

Glucagon is an important counter-regulatory hormone that responds to decreasing blood glucose concentrations. As expected, glucagon release from the α-cells is inhibited by glucose in isolated cells (23), mouse pancreatic α-cells(24) or insulin in mouse pancreatic α-cells (24). In support, glucokinase is also present in the α-cells (25) and, similar to the β-cells, links glucose metabolism to K_{atp} channel closure and subsequent depolarization (26). Intriguingly, this mechanism has an inhibitory role on glucagon secretion (26,27) whereas it stimulates insulin release. Although currently an area of intense interest, much remains to be revealed on the mechanisms of glucagon secretion.

Major Glucoregulatory Tissues

Hepatocytes

The liver is responsible for maintaining blood glucose concentrations during a fast and storing glucose during the postprandial period. This role is central to whole-body glucose homeostasis. The liver is one of the few tissues that express glucose 6 phosphatase (G6P), which removes a phosphate from glucose 6 phosphate creating

free glucose (28); this allows glucose to be released into circulation during a fast. Alternatively, skeletal muscle does not express this enzyme. Glycogen stored within the skeletal muscle must be metabolized in the myocyte and is not available for other tissues. In addition, hepatocytes have first pass access to the glucose absorbed in the gut via the hepatoportal circulation. Similar to the pancreas, the GLUT-2 transporter (29) and glucokinase (2) are expressed in the liver. Due to the kinetic properties of these enzymes (High K_m and High V_{max}) the liver clears a significant portion of the glucose from a meal while it is in portal circulation (28).

Postprandially, insulin is the primary circulating hormone. Because insulin is secreted directly into the portal circulation, a large portion of the insulin is also cleared by the hepatocytes (16). Although glucose transport into the hepatocyte is insulin-independent, insulin is important in many aspects of glucose metabolism in these cells. Circulating insulin interacts with the extracellular α-subunits of its receptor causing the intracellular β-subunits to dimerize and autophosphorylate on tyrosine residues (30). Phosphorylation of the β-subunits permits the docking of the insulin receptor substrates (IRS) 1 and 2 and activation of phosphoinositol 3 kinase PI3K (31). PI3K binds the IRS protein and phosphorylates Protein kinase B (PKB, also known as Akt) and protein phosphatase-1 (PP-1), in the hepatocytes (31). PKB regulates glycogen synthase (GS), the rate-limiting step in glycogen synthesis by phosphorylating and inactivating glycogen synthase kinase 3 while PP-1 activates GS by removing a phosphate (28). PKB also suppresses gluconeogenesis by phosphorylating the forkhead box protein O1 (FOXO1) which then migrates out of the nucleus reducing transcription of the key gluconeogenic enzymes G6P and phosphoenolpyruvate carboxykinase (PEPCK) (28). Coupled with

insulin's depression of glucagon release at the pancreas, insulin has a profound impact on the uptake of glucose for storage and the prevention of its release from the hepatocyte. These actions greatly reduce glucose concentrations in the blood. Although glucose transport into the hepatocyte is insulin-independent, insulin is important in many aspects of glucose metabolism in these cells. Hepatic glucose metabolism is depicted in figure 1 (28).

Glucagon is elevated during a fast and opposes the actions of insulin to promote the release of glucose from hepatocytes. In the hepatocytes, glucagon binds its G-protein coupled receptor stimulating adenylate cyclase and the production of cyclic adenosine monophosphate (cAMP) (32). In response to rising cAMP concentrations, protein kinase A (PKA) phosphorylates and activates glycogen phosphorylase which is responsible for glycogenolysis and gluconeogenesis in the hepatocyte (32). Further, glucagon stimulated PKA the expression of G6P and PEPCK for greater abundance of the enzymes for gluconeogenesis (28). These processes stimulate glucose release into the blood to maintain normoglycemia.

Skeletal Muscle

Skeletal muscle is another important tissue in the regulation of glucose metabolism. Skeletal muscle is a large reservoir for glucose due to its high capacity to synthesize glycogen and its absolute tissue mass (33). After a meal, insulin stimulates glucose transport and storage into skeletal muscle. Unlike the liver, the primary glucose transporter in the skeletal muscle is the insulin-dependent GLUT-4 (34). GLUT-4 proteins are sequestered in intracellular vesicles and translocate to the sarcolemma in

response insulin (31,34). Insulin binding to its receptor leads to activation of PI-3K and subsequently PKB, which stimulates GLUT-4 translocation (35). Glucose is phosphorylated by hexokinase II to form G6P (36), which prevents its escape from the myocyte and allosterically stimulates GS (37,38). Insulin increases the activity and protein content of hexokinase II (39) which further supplements glucose uptake. In addition, insulin stimulated PKB activation also leads to greater GS activity (40). Collectively skeletal muscle is primed to store the glucose from the blood in response to a meal.

Adipose tissue

In adipocytes, opposing insulin's action, glucagon acts to stimulate lipolysis during a fast. Glucagon activates adenylate cyclase stimulating the formation of the second messenger cAMP, increased PKA activity, and phosphorylation of hormone-sensitive lipase (HSL) (10,32). HSL activity leads to the release of NEFAs into the circulation.

In adipocytes, insulin stimulates glucose uptake, triglyceride synthesis, and prevents lipolysis. Similar to skeletal muscle, insulin stimulates glucose transport through the translocation of GLUT-4 to the cell membrane (10). Glucose is stored in adipose primarily as glycerol on triglycerides (10) although not in large quantities. Perhaps the most important action Insulin's primary action on the adipocytes is to block lipolysis, primarily by inhibition of hormone-sensitive lipase (41); this occurs in part by activation of phosphodiesterase, the enzyme that converts cAMP to AMP (42). These actions prevent the release of NEFAs in the blood.

Impaired Glucose Metabolism

Insulin resistance

Insulin resistance is a hallmark of type 2 diabetes and is characterized by a failure of insulin to stimulate an appropriate reduction in blood glucose levels. Importantly, significant insulin resistance can exist without the presence of overt diabetes. A detailed discussion of the mechanisms of insulin resistance and impairments to the insulin signaling pathway is not warranted here, but readers are directed to the following reviews (43–45). In adipose tissue, insulin resistance leads to poor regulation of lipolysis and high postprandial circulating NEFAs; high NEFAs cause insulin resistance in the hepatocytes and skeletal muscle (46). In hepatocytes, the primary defect is the inability of insulin to prevent the release of glucose into the blood (28). Continued hepatic glucose release coupled with a lack of uptake leads to a profound extension of the glucose excursion during the postprandial period. Additionally, in skeletal muscle, disruption of the insulin signal prevents GLUT-4 translocation and glycogen synthesis (44). Altogether, these impairments lead to significantly longer glucose excursions and hyperglycemia. With hyperglycemia, insulin secretion becomes chronically elevated in the early stages of diabetes. Hyperinsulinemia is a very strong predictor of the onset of diabetes and may play a pathogenic role in the progression of the disease (47). In early stages of insulin resistance, the β cells are capable of responding with adequate insulin, but as this worsens the β-cells decompensate and fail to keep up; this marks the development of overt diabetes (48).

Insulin Signaling in the Endothelium

An important component of insulin-mediated glucose uptake into skeletal muscle is insulin's ability to recruit microvascular blood flow to the skeletal muscle (49,50). Insulin recruitment of blood flow to the muscle is through PKB-dependent production of nitric oxide (51,52). Nitric oxide (NO) is an important regulator of blood flow to skeletal muscle (53). Nitric oxide is produced by nitric oxide synthase (NOS) of which there are three isoforms: Inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) (54). eNOS and nNOS are also referred to as constitutive or calcium-dependent NOS (cNOS); these isoforms are constitutively expressed in mammalian tissues (54). NOS catalyzes the reaction that converts molecular oxygen and the amino acid l-arginine to NO and l-citrulline. In order for this reaction to proceed, the cofactor 6R-5,6,7,8-tetrahydrobiopterin (BH4) is required and is an important regulator of NO production (55).

Cyclic guanylyl monophosphate (cGMP) is the second messenger responsible for initiating the cascade of nitric oxide signaling (56). This second messenger is formed from guanosine triphosphate (GTP) by the soluble guanylyl cyclase (sGC) (57). The primary effector of the physiological response to nitric oxide is the cGMP-dependent protein kinase (PKG) (58). In the smooth muscle of the vasculature, PKG activates myosin light chain phosphatase and reduces intracellular calcium concentrations, which leads to smooth muscle relaxation and vasodilation (figure 2. from (53)) (for a detailed review see 54). Insulin mediated vasodilation recruits blood flow to skeletal muscle for storage. Additionally, in skeletal muscle, nitric oxide stimulates the phosphorylation of AMP-dependent protein kinase (AMPK) and greater

uptake of glucose through an insulin-independent mechanism (59). Nitric oxide can also posttranslationally modify proteins in a process called S-nitrosylation. S-Nitrosylation refers to the addition of a NO group to a cysteine residue on target proteins (60). For glucose homeostasis, nitrosylation of GLUT-4 vesicles facilitate greater trafficking to the sarcomere in the skeletal muscle (61), and increases glucokinase activity in the β cells of the pancreas (62). In total, nitric oxide signaling facilitates glucose uptake through both insulin-dependent and insulin-independent mechanisms into skeletal muscle during the postprandial period. Inhibition of NOS diminishes insulin-mediated glucose uptake in rat skeletal muscle (63,64). Not surprisingly, poor NO bioavailability is a common feature in diabetes (49,65). In support, patients with type 2 diabetes insulin fails to stimulate skeletal muscle NO production unlike their healthy counterparts (66). BH4 and L-arginine have both been implicated in reduced NO bioavailability.

Bioavailability of L-arginine in some cases may limit NO synthesis. In endothelial cells, arginase 1 and 2 catalyze the formation of ornithine from arginine (67) effectively reducing arginine's availability for the NOS enzyme. This is supported by findings that arginase 1 activity is increased in human coronary arterioles (68) and in the plasma (66) of type 2 diabetics. Correspondingly, it has been shown that arginase 1 activity is positively correlated with impaired vasodilation in diabetic mice (69,70), yet during a hyperinsulinemic euglycemic clamp, insulin reduced arginase activity in type 2 diabetics and healthy controls, but was unable to increase NO production in type 2 diabetics compared with an approximate doubling in normal controls (66). These findings

suggest L-arginine may not be limiting in human diabetes; this is supported by the indefinite results of arginine supplementation (49).

A loss of NO bioavailability can also occur when the cofactor BH4 is limiting. When BH4 concentrations are insufficient, NOS becomes uncoupled and leads to superoxide generation rather than NO (71–73). Superoxide can further react with NO to form the highly reactive peroxynitrite leading to further degradation of the intracellular NO and BH4 pools (74). BH2 is the product of oxidized BH4 and can begin to accumulate in cells with unusually high oxidative stress (74). In fact, the ratio of BH4 to 7,8 dihydrobiopterin (BH2) is an important mediator of NO synthesis due to competitive BH2 binding by the NOS enzyme (71). In rats with fructose-induced endothelial dysfunction, BH4 administration restored NO-dependent vasodilation in a similar manner to the NO donor sodium nitroprusside (75). Further, the pharmaceutical analog for BH4, sapropterin improves blood flow to human skin in an NO-dependent manner (73). In diabetic rodent models, BH4 supplementation suppresses NOS-dependent hepatic gluconeogenesis and reduced blood glucose during a glucose tolerance test (76). These findings suggest that supplementation with BH4 may be attractive therapeutic option for increasing NO bioavailability. In humans however, oral BH4 treatment may lack efficacy due to oxidation while in circulation (77).

Insulin secretion is also responsive to NO signaling. Insulin secretion is both inhibited and stimulated by NO in the β-cell in a concentration-dependent manner. At high concentrations insulin secretion is depressed, but when the concentration is below 50 nM insulin secretion is stimulated (78,79). The stimulatory action on the β-cells is mediated, in part, by PKG-dependent phosphorylation and inactivation of the K_{atp}

channel (79). At high concentrations, NO appears to blunt glucose metabolism at phosphofructokinase and the mitochondria (80). Further, NOS co-localizes with the insulin granules of the β-cell and is activated by increased intracellular calcium concentrations (81). Similarly, glucokinase also associates with insulin granules; when glucose concentrations rise glucokinase dissociates from the insulin granules and has greater enzymatic activity (82) facilitated by the post translational modification of glucokinase by S-nitrosylation leading to its dissociation from the secretory granule (62).

Dietary Nitrate as a Nutriceutical

Recently, dietary nitrate has gained considerable interest as a means to improve NO bioavailability in symptomatic and healthy subjects independent of NOS. Dietary nitrate is available in most vegetables and is highly concentrated in beets, cured meats, and spinach (83). In the oral cavity, there are bacteria that possess the enzyme nitrate reductase which convert nitrate to nitrite (84–88). Nitrite produced in the oral cavity is then either further reduced to NO in the acidic environment of the stomach (88,89) or absorbed as nitrite (87). Once in circulation, the mechanisms of nitrite bioactivation are still relatively unclear, but several potential interactions have been proposed including interactions with hemoglobin, myoglobin, and carbonic anhydrase (90), and circulation to the salivary glands for excretion into the oral cavity for additional bacterial reduction (88). The importance of oral nitrate reduction is highlighted by the ability of antibacterial mouthwash to lower the plasma nitrite response to nitrate ingestion (87). Regardless, dietary nitrate increases the bioavailability of NO in a variety of models, including humans.

With respect to diabetes, numerous models have been used to study the effects of dietary nitrate on glucose homeostasis. Dietary sodium nitrate has been shown have positive effects in eNOS-deficient mice. In this model sodium nitrate ingestion significantly reduced the glucose excursion after an intraperitoneal glucose tolerance test compared with water (91). The blood pressure in these mice was also reduced following dietary nitrate administration. Addition of the NOS inhibitor N (G)-nitro-L-arginine methyl ester (L-NAME) increased blood pressure in both groups, but the dietary nitrate group resisted this increase for several hours indicating a substantial role for dietary nitrate in generating NO independent of the NOS pathway in these mice (91). In human participants with elevated risk of cardiovascular disease a 30-day dietary nitrate supplementation increased plasma nitrite and nitrate concentrations (92). Plasma nitrite is associated with improved glucose homeostasis in rodents (91) and increased cGMP concentrations and lower blood pressure in humans (93). Moreover, potassium nitrate reduced the area under the insulin and glucose curves during an oral glucose tolerance test in healthy subjects (94).

In opposition, beet juice ingestion has also been shown to have no effect on blood pressure or insulin sensitivity in patients with type 2 diabetes (95). In this study, subjects were given either 250mL of beet juice or placebo for two weeks. However, these subjects were tested after an overnight fast. These data suggest that short-term supplementation with dietary nitrate may be insufficient to elicit long-term adaptation. Alternatively, due to the rapid metabolism of the NO, the beneficial effects of dietary nitrate ingestion could be limited to the postprandial period. Indeed, a recent meta-analysis assessing both supplementation and acute treatments with dietary nitrate

found that inorganic nitrate/beet juice ingestion to be significantly associated with reduced blood pressure in humans (96). Despite this, the potential therapeutic benefit of nitrate ingestion on glucose homeostasis remains unclear. There is currently a great deal of evidence supporting a role of acute dietary nitrate metabolism improving the NO bioavailability in humans. Moreover, NO bioavailability has been shown to have a large role in the pathology of type 2 diabetes. Still, more studies are needed to clarify the potential for dietary nitrate to acutely improve glucose tolerance.

Hypothesis.

NO is an important physiological determinant of glucose tolerance.

Specific aim.

To determine if acute dietary nitrate administration augments glucose disposal.

CHAPTER II

INTRODUCTION

Type 2 diabetes mellitus is associated with impaired macronutrient metabolism and vascular dysfunction. The primary metabolic defect is decreased insulin sensitivity resulting in elevated fasting blood glucose and insulin values (47). It has been shown in rodents that increased microvascular perfusion is an early event in insulin-mediated glucose disposal, occurring within minutes; an effect regulated by nitric oxide dependent processes (50). Indeed, nitric oxide (NO) -dependent vascular control has been shown to be impaired in type 2 diabetes mellitus (97). Moreover, in rodent skeletal muscle, inhibition of Nitric Oxide Synthase (NOS) completely suppressed the insulin-mediated recruitment of the microvasculature and dramatically lower glucose disposal in response to insulin (63,98). Dietary nitrate has recently gained interest as a means to improve NO bioavailability. Dietary nitrate is reduced by bacteria possessing the enzyme nitrate reductase in the oral cavity reducing salivary nitrate and nitrite to nitrite and NO, respectively (88). Nitrite can also be reduced in the stomach to NO due to the low pH (89). In support, humans using antibacterial mouthwash prior to nitrate ingestion have significantly less salivary and plasma nitrite than when they consumed nitrate alone (87). Additionally, dietary nitrite leads to nitrosylation of cysteine residues on GLUT-4, leading to GLUT-4 incorporation in the membrane (61). Dietary nitrate also normalized glucose tolerance and fasting blood glucose in eNOS-deficient mice (91).

Improvements to NO bioavailability have promising therapeutic value for patients with impaired glucose tolerance. Specifically, dietary nitrate could provide an accessible and affordable alternative to medications. Therefore, the purpose of this investigation is to determine the influence of dietary nitrate on glucose disposal during an oral glucose tolerance test (OGTT) in humans. We hypothesize that NO is an important physiological determinant of glucose tolerance.

CHAPTER III

METHODS AND MATERIALS

Research Participants.

Research participants were 10 (2 male, 7 female) sedentary, apparently healthy, overweight or obese adults. Study exclusion criteria consisted of fasting blood glucose concentration ≥ 100 mg/dL, pregnancy, regular use of tobacco products, and medications that might confound the interpretation of data such as blood pressure lowering medications or antihyperglycemic agents. The experimental protocol conformed to the standards set by the Declaration of Helsinki of 1975, as revised in 1983, and was approved by the Institutional Review Board at Colorado State University. The nature, purpose and risks of the study were explained to each research participant before written informed consent was obtained.

Experimental Design.

On completion of health screening and baseline testing, research participants reported to the laboratory after an overnight fast. Participants refrained from dental hygiene for the previous 12 hours, including gum chewing and avoided foods known to contain nitrates such as leafy green vegetables, beets, and cured meats for 24 hours prior to their visit. Upon arrival to the laboratory, a Teflon catheter was placed in an antecubital vein for repeated blood sampling. Using a crossover design, four oral

glucose tolerance tests (OGTT) were performed with a 7-day washout period. To assess the influence of dietary nitrate, subjects consumed either 500mL of beet juice + 25g glucose, or 500mL of water + 75g glucose, with (Beet Juice, Water) and without prior antibacterial mouthwash use (Beet Juice + MW, Water + MW). Beet juice was selected due to its high concentration of nitrate. Mouthwash conditions are intended to blunt the reduction of nitrate to nitrite in the oral cavity

Oral Glucose Tolerance Testing

Glucose tolerance was determined in response to 500 mL of organic beet juice (Biotta; CAJ food products; Carmel, IN) sweetened with 25 grams of dextrose or 500 mL of water sweetened with 75 grams of dextrose. The carbohydrate load for each condition was approximately 75 grams total. During mouthwash trials subjects first rinsed with 10 mL of a hydrogen peroxide mouthwash (1.5% H_2O_2 Peroxyl; Colgate Oral Pharmaceuticals, Inc., NewYork, NY) for one minute followed by two, one-minute rinses with 10 mL of an antibacterial mouthwash (chlorhexidine digluconate; Corsodyl, BCM Ltd., Nottingham, UK). Subjects then consumed their carbohydrate beverage and remained supine for the duration of testing.

Blood Sampling.

Blood glucose was determined on arrival and at the following time-points 5, 10, 20, 30, 45, 60, 90, and 120 minutes using a blood glucose analyzer (2300 Stat Plus, Yellow Springs Instruments, Yellow Springs, Ohio). Blood Samples were collected

upon arrival and at time-points 10, 20, 30, and 120 minutes and placed into serum separator tubes for insulin analysis. Samples were centrifuged within 30 minutes and serum was immediately separated into 1mL aliquots. Serum was stored at -80° C for subsequent analysis. Insulin was determined using a commercially available ELISA kit (Alpco; Salem, NH).

Statistical Analyses.

This was a randomized crossover design with repeated measures, thus we examined the influence of dietary nitrate on blood glucose and serum insulin using a two-way repeated measures ANOVA (beet juice *vs.* water, mouthwash *vs.* no mouthwash). The area under the curve (AUC) for glucose and insulin were determined using the trapezoidal method and then analyzed using a two-way repeated measures ANOVA. Additionally, insulin sensitivity was estimated using the Matsuda Index, which is highly correlated (r = 0.73) with the hyperinsulinemic euglycemic clamp (99), using the following equation:

$$\frac{10000}{\sqrt{(FBG \times FSI) \times (mean\ G \times mean\ I)}}$$

Where:

FBG = fasting blood glucose FSI = fasting serum insulin

Mean G = two hour glucose mean Mean I = two hour insulin mean

These estimations of insulin sensitivity were compared by two-way repeated measures ANOVA. The level of statistical significance was set at $P < 0.05$. Data within text and tables are expressed as mean ± SE.

CHAPTER IV

RESULTS

Ten subjects were enrolled in the experiment, but one participant's results were excluded from the analysis due to substantially high fasting blood glucose (>126 mg/dL). Nine subjects completed all four conditions of the study without any adverse events and are included in all analyses. The remaining participant's fasting blood glucose concentrations were in the healthy range, <100 mg/dL. Participants were assessed for body composition using dual-energy x-ray absorptiometry (DXA-IQ; Lunar Radiation corp., Madison, WI, software version 4.1). Selected physiological characteristics are presented in Table 1.

Acute Dietary Nitrate and Blood Glucose

Circulating glucose responses are presented in figure 3. Acute dietary nitrate ingestion does not influence the circulating glucose concentrations during an oral glucose tolerance test (figure 3.). However, both Beet Juice conditions had a modestly lower glucose AUC, 9% (figure 4) and the final glucose concentration (figure 5) was 17% lower in the Beet Juice and Beet Juice + MW conditions as compared with the Water conditions (main effect of beet juice, $P < 0.05$). Intriguingly, there was also a main effect of mouthwash resulting in an approximate 6% lower glucose AUC and 9% lower circulating glucose at 120 minutes compared with the no mouthwash conditions,

figures 4 and 5, respectively ($P < 0.05$). The differences in peak glucose failed to attain significance at $P = 0.08$.

Acute Dietary Nitrate and Serum Insulin

Circulating insulin levels were not different between conditions (figure 6). Insulin AUC in both Beet Juice conditions was lower compared with the Water conditions (main effect of Beet Juice. $P < 0.05$). Peak insulin was 18% lower in Beet Juice compared with Water ($P < 0.05$).

Insulin Sensitivity

The results of the Matsuda Index calculations are presented in Figure 8. The Matsuda Index was approximately 25% greater in the Beet Juice condition than the Water condition ($P < 0.05$ *vs.* water) indicating greater insulin sensitivity. There were no other interactions or main effects for the Matsuda Index.

CHAPTER V

DISCUSSION

The primary findings of the current investigation are: 1) Estimated insulin sensitivity is greater when 75g of carbohydrate is consumed in beet juice compared with 75g of carbohydrate in water. 2) Improved insulin sensitivity is evidenced by reduced area under both the glucose and insulin curves when 75g of carbohydrate is consumed in beet juice.

To our knowledge, we are the first to evaluate the influence of acute dietary nitrate intake on insulin sensitivity in an obese population. Central to the strength of our study design is the timing of the nitrate ingestion in a meal-like setting within a naturally occurring food source. This design accounts for the short half-life of NO in circulation by delivering the nitrate concurrently with the carbohydrate. We hypothesized that dietary nitrate is the primary bioactive component in beet juice that improves insulin sensitivity, however, beet juice is rich in many nutrients and phytochemicals. To evaluate the effect of the nitrate in the beet juice, we used an antibacterial mouthwash to attenuate the reduction of nitrate to nitrite. Importantly, the nitrate load was the same in both Beet Juice conditions.

Plasma nitrite concentrations and time-to-peak concentrations increase dose-dependently for several hours following beet juice ingestion (100). Consumption of 280 mL of beet juice elicits an approximate four-fold increase in plasma nitrite after one hour, which rises to a seven-fold increase after two hours (100). Additionally, no further

lowering of blood pressure is detected when volumes of beet juice greater than 140 mL are consumed (100). Therefore, the volume of beet juice consumed in this study, 500 mL, is expected to be sufficient to elicit a physiological response.

We witnessed a lower postprandial glucose excursion with Beet Juice that persisted for two hours. In order to explain these observations we considered the specific carbohydrate composition of the carbohydrates in the beverages. The main effect of Beet Juice on the glucose AUC and 120 minute glucose values indicates improved glucose tolerance when 75g of carbohydrate is consumed in beet juice compared with 75g of carbohydrate in water. However, approximately two-thirds of the carbohydrate in the Beet Juice trials was sucrose (101), which is catalyzed into glucose and fructose by sucrase-isomaltase located in the brush border of the intestinal mucosa (102). Fructose metabolism in humans is quite different than glucose in that fructose is primarily cleared by the liver and does not elicit a significant insulin response (103). This fact may explain a portion of our current findings, but there was not a main effect of Beet Juice on insulin sensitivity indicating other mechanisms. A main effect of Beet Juice on the final glucose concentration is clinically relevant in that this test is often used to diagnose diabetes. It has been suggested that peripheral insulin resistance is the primary cause for blunted responses during an OGTT (99). Our results indicate that beet juice likely influences the uptake of glucose into skeletal muscle.

In the current investigation Beet Juice, and potentially dietary nitrate, was associated with a lower postprandial insulin excursion. With respect to insulin secretion, nitric oxide is stimulatory at lower concentrations, but inhibitory at greater concentrations (80). Possibly, blood nitrite concentrations in the Beet Juice condition

were sizeable enough to inhibit insulin secretion, an effect diminished in the mouthwash condition. This is supported by our findings that insulin secretion in the Beet Juice was significantly lower compared with Water, but the Beet Juice + MW condition was not. Inhibition of insulin secretion could be an important aspect of how dietary nitrate regulates glucose metabolism. The lower area under the glucose curve could also account for this observation due to reduced glucose stimulated insulin secretion in β-cells.

The Matsuda Index incorporates fasting and postprandial measures of glucose and insulin in order to more accurately depict insulin sensitivity (99). We found that estimated insulin sensitivity was significantly greater in the Beet Juice trial when compared with the Water trial. However, the Beet Juice + MW trial was not different from Water suggesting that dietary nitrate could have influenced insulin sensitivity, but the comparison within the Beet Juice conditions was not significant (P=0.16). To verify that mouthwash had no impact on insulin sensitivity, we included the water conditions. Unexpectedly, we found that mouthwash significantly lowered the glucose AUC and the final glucose concentration. The presence of carbohydrate sensors in the oral cavity (104) could explain this phenomenon, possibly through enhanced sweet taste sensitivity or greater access to the sensors, but this cannot be confirmed in the present study. However, the glucose response to mouthwash may be somewhat masking the effects of dietary nitrate on the estimates of insulin sensitivity.

We are not the first to investigate the influence of dietary nitrate on insulin sensitivity. Recently, acute dietary nitrate was found to improve (94) and have no effect (105) on glucose tolerance. Acute potassium nitrate supplementation reduced the

glucose AUC and increased the insulin response to an OGTT in healthy males (94). However, in these experiments the nitrates were administered to participants in the healthy weight range compared with the obese subjects in our study. Alternatively, our results suggest that dietary nitrate blunts the insulin response.

Conclusion

Beet Juice improves estimates of insulin sensitivity, partially mediated by a dramatic reduction of the insulin response to a carbohydrate challenge. Importantly, our data suggest that consuming nitrate within the context of a meal is an important timing consideration when attempting to influence glucose metabolism. While we selected beet juice for the present investigation, many vegetables are rich in nitrate. Vegetables are an important component of a nutritious diet and our findings may have revealed another important benefit of vegetables.

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APPENDIX I

TABLES AND FIGURES

Table 1. Subject characteristics n=9 (2 males)

Variable	Baseline
Age (years)	45 ± 4
Body mass (kg)	90.1 ± 10.9
Height (m)	1.65 ± 0.02
Body mass index (kg/m^2)	33.7 ± 0.89
Percent body fat	43.2 ± 5.3
Fat free mass (kg)	51.4 ± 2.4
Fat mass (kg)	38.8 ± 1.6

Data: mean ± SE.

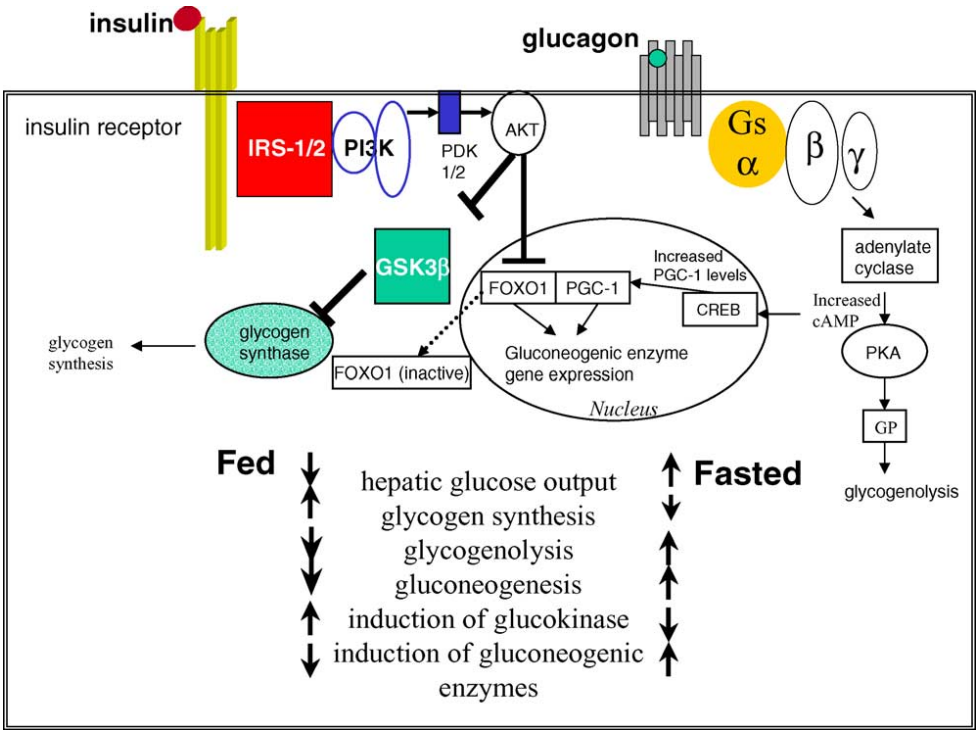

Fig. 1. Summary of glucose homeostatic pathways in the hepatocyte. In the fed state, the insulin-activated receptor signals primarily through tyrosine phosphorylation of its substrates, IRS-1/2. Tyrosine phosphorylated IRS-1/2 associate with and activate signaling intermediates, particularly phosphatidylinositol-3-kinase, which regulate downstream metabolic endpoints. The net effect promotes glucose utilization and inhibits glucose output via regulation of enzyme activity and gene induction. In the fasted state, glucagon signals through its G protein-coupled receptor to regulate metabolic endpoints which promote glucose output and suppress glucose utilization.

Figure 1. ⊥ = Block/inhibit. ↓ = Stimulate/activate (28)

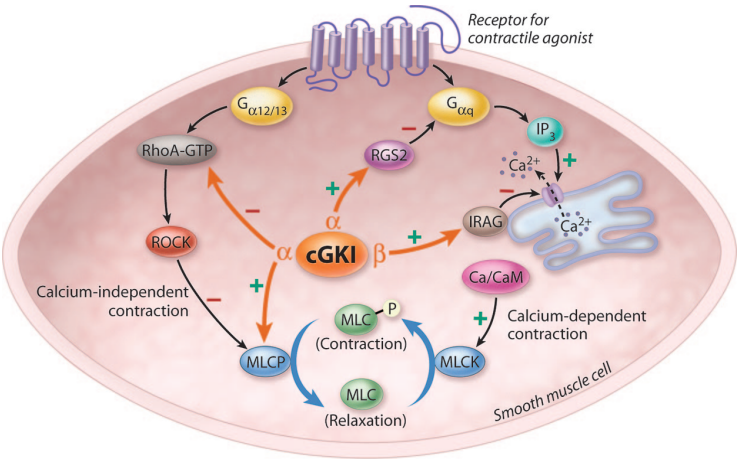

Figure. MLC phosphorylation determines smooth muscle contractility. Contractile agonists lead to inositol 1,4,5 triphosphate (IP_3) production or activation of RhoA (RhoA-GTP). IP_3 binding to its receptor in the sarcoplasmic reticulum leads to release of Ca^{2+}. Ca^{2+}/calmodulin binds to and activates MLCK, which in turn phosphorylates MLC (calcium-dependent contraction). Activated RhoA binds to and activates ROCK, leading to phosphorylation and inhibition of MLCP, inhibiting MLC dephosphorylation (calcium-independent contraction). cGKI mediates relaxation by inhibiting both calcium-dependent and -independent contraction. cGKIα activates MLCP by a direct interaction and by inhibition of RhoA, and activates RGS2 to inhibit $G_{\alpha q}$ signaling. cGKIβ activates IRAG, which then inhibits Ca^{2+} release by the IP_3 receptor.

Figure 2. Myosin Light Chain phosphorylation determines smooth muscle contractility (53).

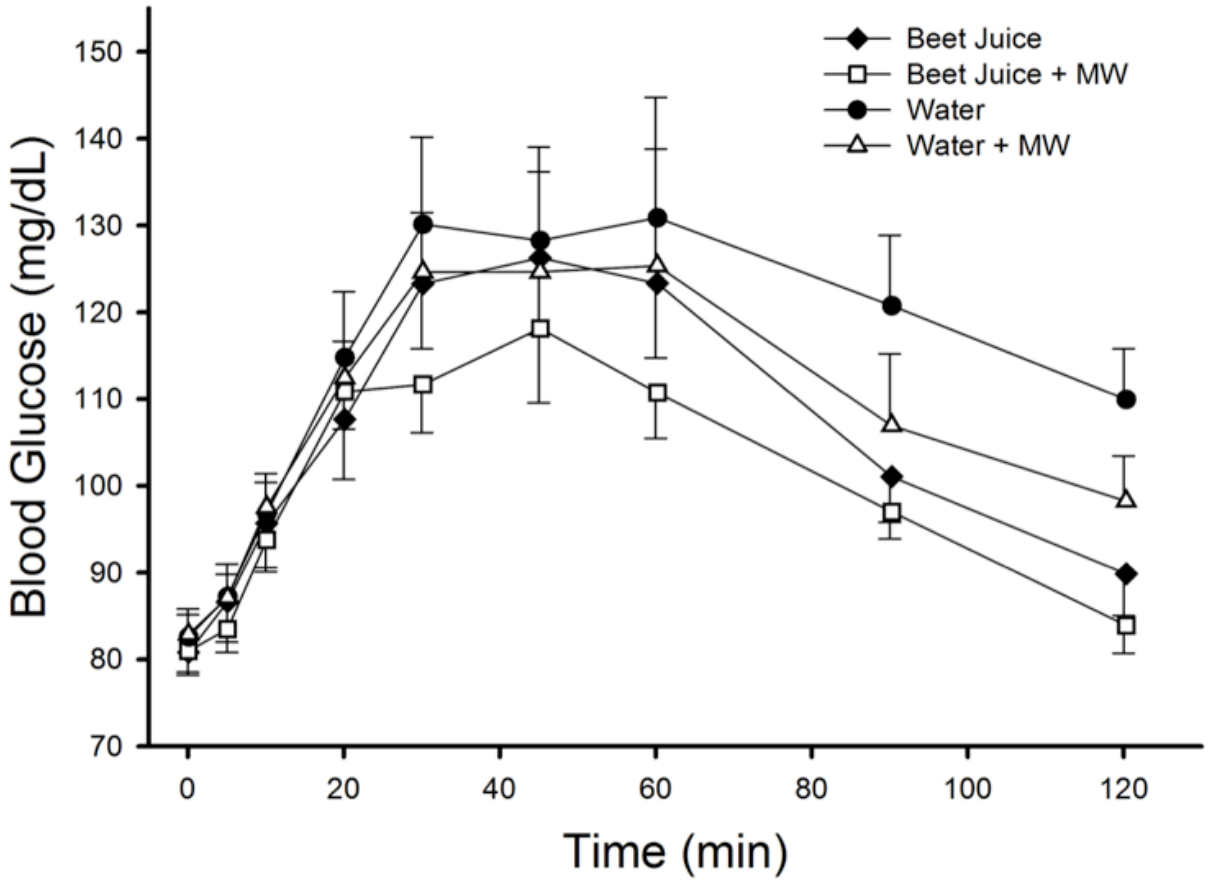

Figure 3. Effect of dietary nitrate on circulating glucose concentrations. There was no effect of dietary nitrate on the circulating glucose response to an OGTT. *P* > 0.05. Data: mean ± SE.

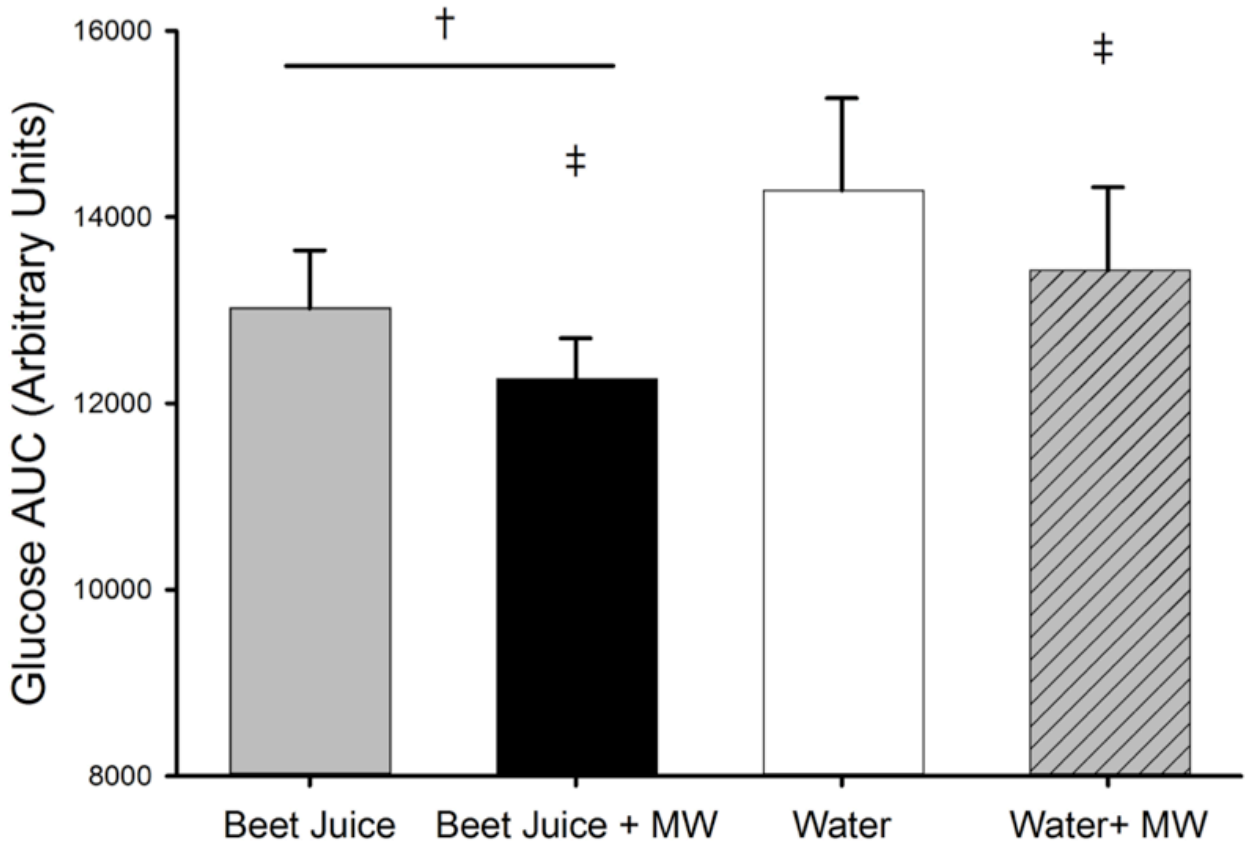

Figure 4. Effect of dietary nitrate on the area under the glucose curve. †P < 0.05 main effect of Beet Juice *vs*. Water. ‡*P* < 0.05 main effect of Mouthwash *vs.* No Mouthwash. Data: mean ± SE.

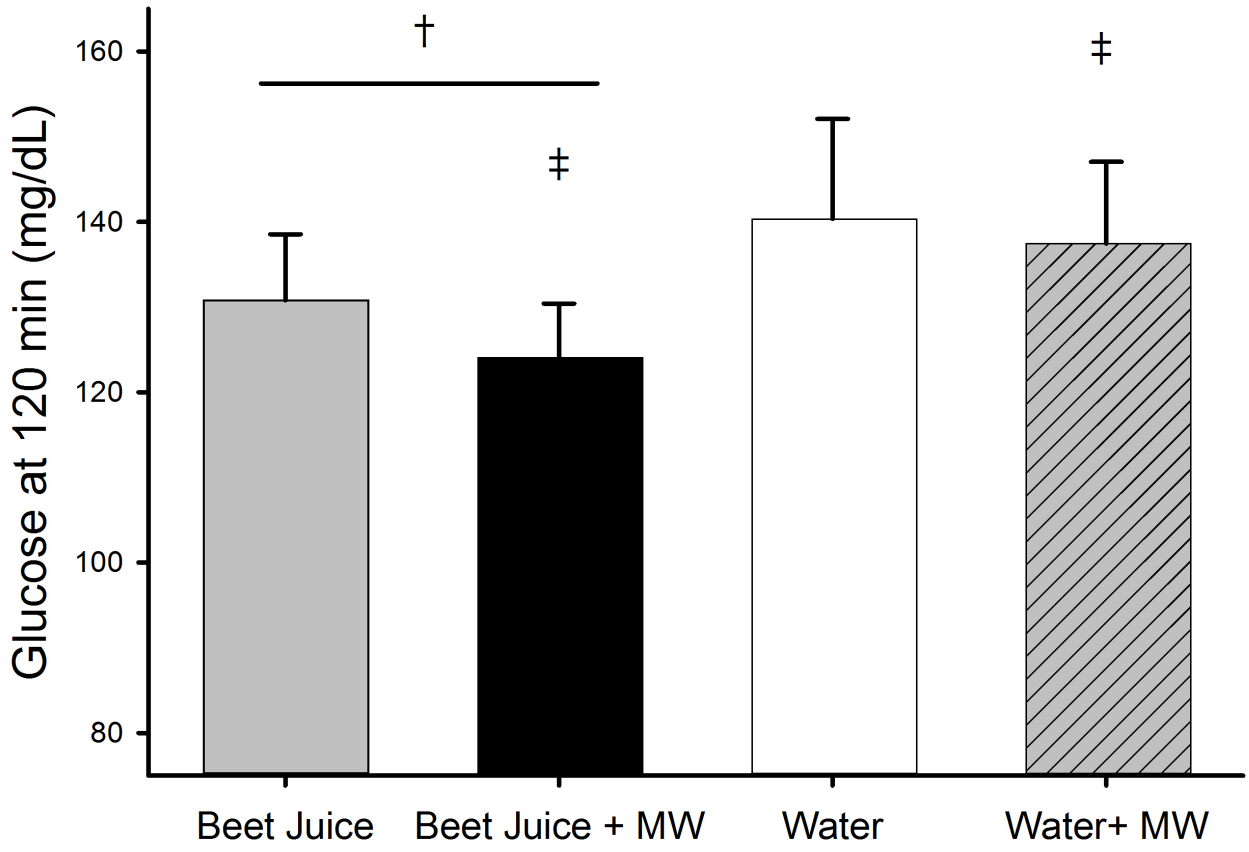

Figure 5. Effect of dietary nitrate on the final circulating glucose concentration. †P < 0.05 main effect of Beet Juice *vs*. Water. ‡*P* < 0.05 main effect of Mouthwash *vs.* No Mouthwash. Data: mean ± SE.

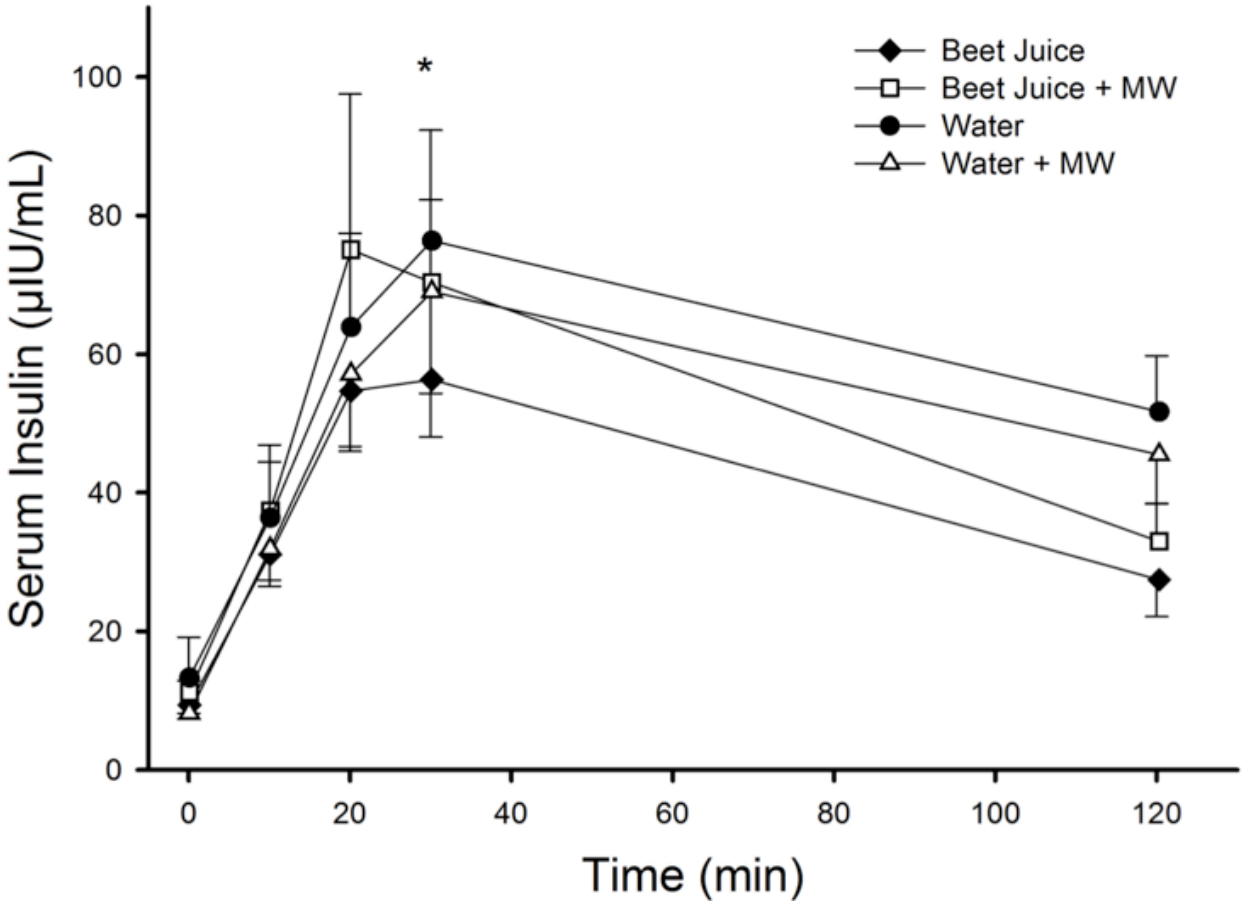

Figure 6. Effect of dietary nitrate on circulating insulin concentrations. There was no effect of dietary nitrate on the circulating insulin response to an OGTT. $P > 0.05$. Peak insulin concentrations were greater in Water compared with Beet Juice. $^*P < 0.05$ Beet Juice *vs.* Water. Data: mean ± SE.

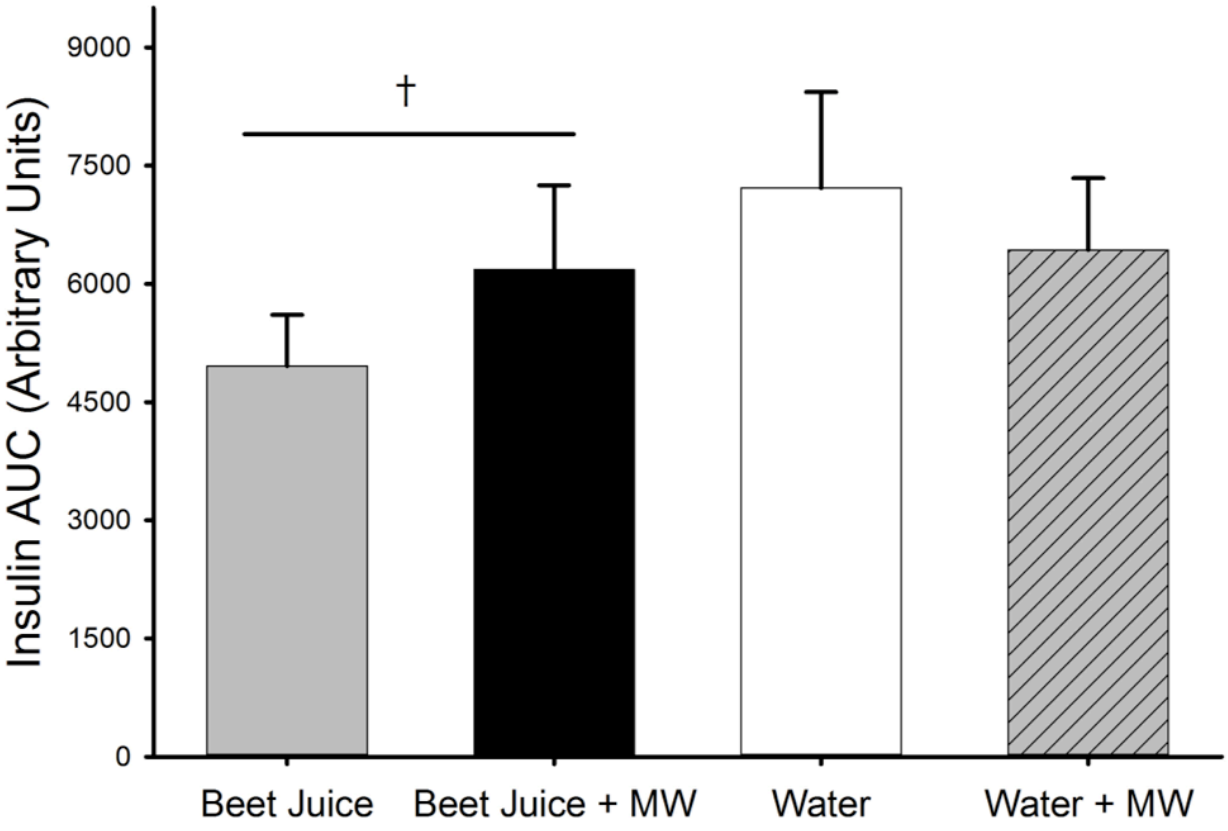

Figure 7. Effect of dietary nitrate on the area under the insulin curve. †P < 0.05 main effect of Beet Juice *vs*. Water. Data: mean ± SE.

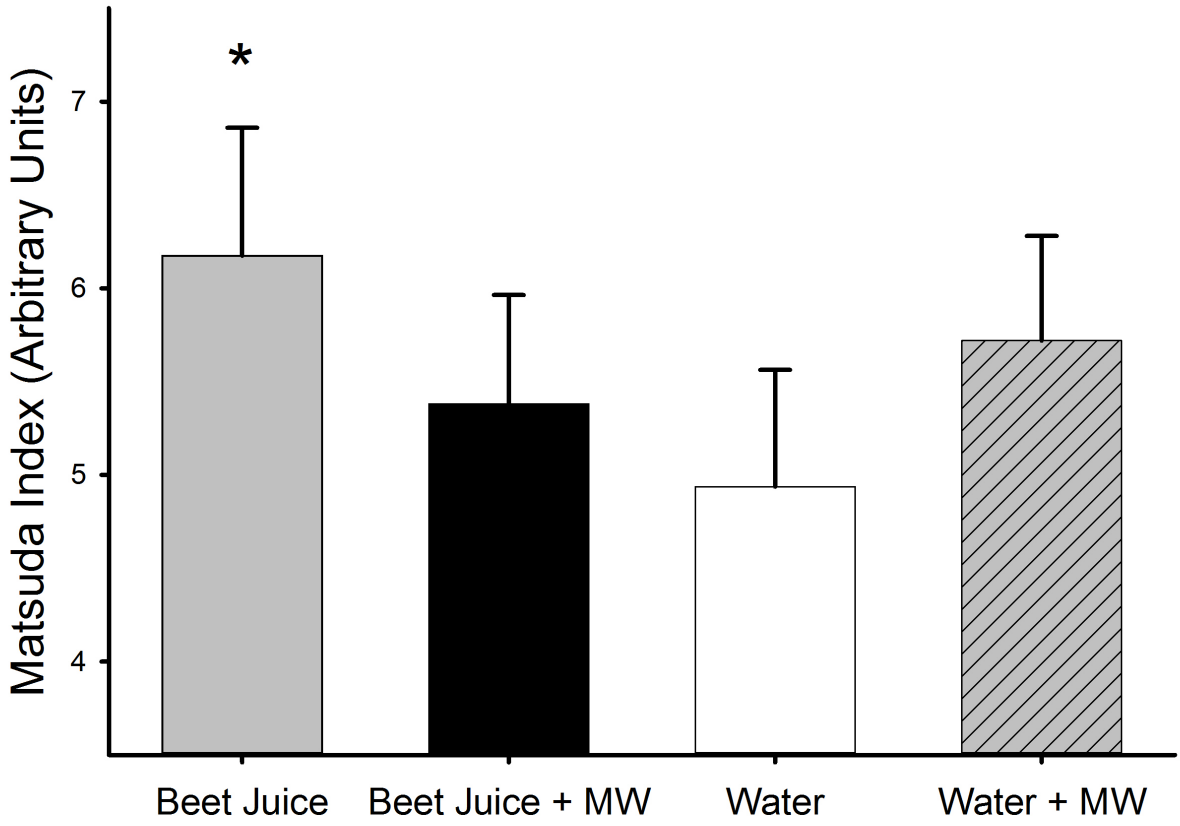

Figure 8. Effect of dietary nitrate on the Matsuda Index of insulin sensitivity. There was no effect of dietary nitrate on the estimated insulin sensitivity. $P = 0.15$. Estimated insulin sensitivity was greater in Beet Juice as compared with Water. $^{*}P < 0.05$ Beet Juice *vs.* Water. Data: mean ± SE.