BIOAVAILABILITY OF DIETARY OXALATE

by

MARY CATHERINE ROBERTSON

SUSAN MILLER, COMMITTEE CHAIR
BETTY DARNELL
ROSS HOLMES
ROBERT OSTER

A THESIS
Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Master of Science

BIRMINGHAM, ALABAMA

2015
Urolithiasis, or kidney stone disease, is a costly and painful health problem affecting about 8.8% of Americans with prevalence on the rise. Calcium oxalate stones account for about 80% of all stones. Increased urinary excretion of calcium and oxalate is a risk factor and central feature of such stones; dietary oxalate contributes up to 50% of urinary oxalate. The purpose of our research is to determine if physical manipulation such as chewing or blending prior to ingestion may render some oxalate more accessible for absorption in spinach, a high-oxalate food. In this crossover, controlled trial we enrolled six healthy, non-stone forming individuals who consumed three different oxalate loads and provided timed urine collections for 24 hours following. The oxalate loads provided 500mg of oxalate and were given via smoothie. One load was a smoothie with blended spinach, one contained spinach cut into 1 cm sq, and one contained soluble sodium oxalate. The results showed that for the six hours following each load, there were significant differences in the amount of oxalate excreted. The load with sodium oxalate had significantly higher oxalate excreted when relative to creatinine than the 1 cm sq spinach (P=0.009) and was trending toward significance when compared to the blended spinach (P=0.052). There were no significant differences between the blended spinach and 1 cm sq spinach or among the three groups for the entire 24-hr period.
# TABLE OF CONTENTS

ABSTRACT ................................................................................................................. ii

LIST OF TABLES ........................................................................................................... v

LIST OF FIGURES ....................................................................................................... vi

LIST OF ABBREVIATIONS ......................................................................................... vii

INTRODUCTION .......................................................................................................... 1

HYPOTHESES AND SPECIFIC AIMS ................................................................. 4

REVIEW OF LITERATURE ......................................................................................... 6

  Kidney Stones ......................................................................................................... 6
  Oxalate ..................................................................................................................... 7
  Variability of Oxalate Content in Foods ................................................................. 9
  Bioavailability of Soluble Versus Insoluble Oxalate ........................................... 10
  Bioaccessibility of Nutrients within Food Matrices .......................................... 10
  Concomitant Foods ................................................................................................. 12
  Effects of Calcium on Oxaluria ............................................................................ 12
  Effects of Food Processing on Oxalate Bioavailability ....................................... 14
  Oxalobacter Formigenes ......................................................................................... 14
  Genetic Differences in Hyperoxaluria ................................................................. 15
  Other Risk Factors of Hyperoxaluria ................................................................. 16
  Summary .................................................................................................................. 18

METHODOLOGY ....................................................................................................... 19

  Subjects .................................................................................................................... 19
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Multivariate relative risks for kidney stones according to category of 24-hr urinary oxalate excretion within NHS I, NHS II, and HPFS</td>
</tr>
<tr>
<td>2</td>
<td>Nutrient composition of study diet</td>
</tr>
<tr>
<td>3</td>
<td>Mean ± SD baseline and post-load urinary volume (mL)</td>
</tr>
<tr>
<td>4</td>
<td>Mean ± SD baseline and post-load urinary creatinine (mg)</td>
</tr>
<tr>
<td>5</td>
<td>Mean ± SD baseline and post-load urinary oxalate (mg)</td>
</tr>
<tr>
<td>6</td>
<td>Mean ± SD baseline and post-load urinary Ox(mg)/Cr(g)</td>
</tr>
<tr>
<td>7</td>
<td>Mean ± SD percent bioavailability of oxalate</td>
</tr>
<tr>
<td>8</td>
<td>Percent of total oxalate extracted (mean ± SD) during in vitro simulated digestion</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
</tr>
<tr>
<td>Comparison of urinary Ox(g)/Cr(mg)</td>
<td>Comparison of cumulative percent bioavailability of oxalate</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT</td>
<td>alanine: glyoxylate aminotransferase</td>
</tr>
<tr>
<td>CRU</td>
<td>Clinical Research Unit</td>
</tr>
<tr>
<td>ESRD</td>
<td>end-stage renal disease</td>
</tr>
<tr>
<td>HPFS</td>
<td>Health Professionals Follow-up Study</td>
</tr>
<tr>
<td>IC</td>
<td>Ion chromatography</td>
</tr>
<tr>
<td>NHS I</td>
<td>Nurses’ Health Study I</td>
</tr>
<tr>
<td>NHS II</td>
<td>Nurses’ Health Study II</td>
</tr>
<tr>
<td>1 cm sq</td>
<td>one centimeter squares</td>
</tr>
<tr>
<td>Ox/Cr</td>
<td>oxalate (mg)/creatinine (g)</td>
</tr>
<tr>
<td>%BA</td>
<td>percent bioavailability</td>
</tr>
<tr>
<td>PH1</td>
<td>primary hyperoxaluria type 1</td>
</tr>
<tr>
<td>PH2</td>
<td>primary hyperoxaluria type 2</td>
</tr>
<tr>
<td>PH3</td>
<td>primary hyperoxaluria type 3</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes</td>
</tr>
<tr>
<td>UAB</td>
<td>University of Alabama at Birmingham</td>
</tr>
</tbody>
</table>
INTRODUCTION

The direct and indirect costs of urolithiasis, or kidney stone disease, are immense. In 2000, one study showed the total cost in the U.S. to be $2.1 billion (1). However in more recent years, with rising prevalence and growth of healthcare expense, that cost has been shown to exceed $5 billion (2). Costs have been attributed to emergency room visits, hospitalizations, operative procedures, and patient work days lost (3). The financial and social burdens of kidney stone disease may be reduced by decreasing the prevalence and rates of reoccurrence, which could potentially be achieved by providing improved dietary guidelines and recommendations (1).

Kidney stones are attributed to genetic and environmental traits, and while there are many types of kidney stones, the most common type is calcium oxalate which accounts for about 80% of all stones. Increased urinary oxalate (hyperoxaluria) is a risk factor and central feature of such stones. Oxalate is a strong dicarboxylic organic acid (C$_2$O$_4$H$_2$) that is produced endogenously by the body and consumed in dietary sources. It has been shown that as much as one half of the amount of urinary oxalate can be attributed to dietary oxalate, which is much higher than previously thought (4).

Most oxalate-containing foods are plants, including spinach, rhubarb, chocolate, tea, beets, chard, nuts, and wheat bran (5). Many factors influence the oxalate content of food; the amount can vary within plant species and even within a single plant. This variability can be attributed to many growing conditions such as soil composition, amount of sunlight or other nutrients available during growth, geographic location, rate of growth,
and timing of harvest (6, 7). Oxalate is found within the idioblasts (storage cells) of plants (8) and may be in the form of soluble or insoluble salts. Soluble oxalate can be absorbed in the gastrointestinal (GI) tract and excreted in the urine, or it can bind with divalent cations such as calcium and pass through the GI tract unabsorbed. Insoluble (crystalized) oxalate is less likely to be absorbed due to the tight associations of the molecular constituents. It generally passes through the gastrointestinal tract unabsorbed and is excreted in the feces. This information causes us to question if physical acts upon the idioblasts, such as mastication or blending prior to ingestion, may render some oxalate more accessible for absorption.

There are several factors that may influence oxalate absorption and excretion within and among individuals. In non-stone forming individuals, oxalate absorption is widely variable at a rate of about 1-9%, and stone formers have an even higher absorption rate. Some factors which effect variability of oxaluria include endogenous oxalate synthesis, renal handling of oxalate, colonization with oxalate-degrading bacteria, and the processing or cooking of foods prior to ingestion (9, 10). Another cause for hyperoxaluria is due to genetic variations resulting from an autosomal recessive disease known as primary hyperoxaluria. So far, three types of primary hyperoxaluria have been discovered. A diet low in oxalate and adequate in calcium in order to reduce oxalate excretion in the urine with hopes to decrease the chance of forming stones is recommended for many of these patients (11). Other factors that may increase urinary oxalate and have an association with kidney stone formation are chronic low dietary calcium intake, increased dietary oxalate intake, and increased dietary vitamin C (12-16).
The entire in vivo process of oxalate production and metabolism is still not completely known, and many factors cause variation in oxalate excretion. This makes conducting a clinical trial difficult. However, we hope to control some of these variables and gain insight regarding the bioaccessibility and bioavailability of oxalate to aid clinicians and patients in their goals of reducing kidney stones.
HYPOTHESES AND SPECIFIC AIMS

Hypothesis 1: The bioavailability of oxalate in spinach will be greater when spinach is completely homogenized when compared to spinach cut into one centimeter squares (1 cm sq) because the act of blending food disrupts cell walls and dissociates oxalate crystals to make the oxalate more soluble and therefore more bioavailable.

- Specific Aims: To estimate and compare the bioavailability of oxalate in spinach following varying degrees of homogenization. We will examine an index of oxalate absorption in healthy individuals consuming three different oxalate load meals. The oxalate loads will consist of either soluble sodium oxalate salt or spinach, a high-oxalate food. The spinach will either be completely homogenized or cut into approximately 1 cm sq. All three of the oxalate loads will be delivered via smoothie and will be low in calcium content. We will provide the same initial amounts of oxalate in the load meals. We expect the sodium oxalate salt to yield the maximum absorption of oxalate per individual. This will serve as a control to which we will compare the absorption rate of dietary oxalate in spinach with varying degrees of homogenization. We will analyze urinary oxalate excretion in two-hour increments at baseline, and at two, four, six, and also at 24 hours following the oxalate load to estimate the amount of oxalate absorbed. Through statistical analyses, we will determine if the degree to which spinach is homogenized significantly affects the bioavailability of the dietary oxalate contents.
Hypothesis 2: Simulating digestive processes in vitro will provide insight to in vivo digestive responses of the small intestines after ingesting a known amount of spinach.

- Specific Aims: To estimate how much oxalate is released from the two forms of spinach by simulating an environment similar to the human gastrointestinal tract in vitro. We will subject spinach that has been blended or cut into 1 cm sq to a solution with similar characteristics of the stomach and small intestine. Then we will compare the in vitro degradation that occurs with the in vivo digestion and metabolism resulting from the consumption of the spinach smoothies. These studies will help determine whether maceration or blending of spinach is an important variable influencing oxalate absorption from spinach.
REVIEW OF LITERATURE

Kidney Stones

Urolithiasis, also known as kidney stone disease, is a costly and painful health problem affecting about 8.8% of Americans (17) with prevalence on the rise (18). Kidney stones are attributed to environmental and genetic traits. They are considered part of a systemic disorder that may be associated with obesity, type 2 diabetes (T2DM), metabolic syndrome, bone loss and fractures, coronary artery disease, and hypertension (19, 20). Genetic traits that influence the amounts of calcium, oxalate, and other ions also contribute to the disease. The fact that the prevalence of obesity, T2DM, and metabolic syndrome have all risen dramatically over the past few decades is well-established; like these disorders, the prevalence of kidney stone disease has also been on the rise and has doubled over the past 30 years (20, 21). If left untreated, kidney stones are considered a chronic illness with the chance of reoccurrence within 10 years reaching beyond 50% (20).

While there are many types of kidney stones, the most common type is calcium oxalate which accounts for about 80% of all stones. Increased urinary excretion of calcium and oxalate (hypercalciuria and hyperoxaluria, respectively) are risk factors and central features of such stones (4).
Oxalate

Oxalate is a strong dicarboxylic organic acid (C$_2$O$_4$H$_2$) that is produced endogenously and consumed in dietary sources. Although the complete pathways that lead to endogenous oxalate synthesis are virtually still unknown, current research is being conducted to better understand the complete process of oxalate production (22). It is widely agreed that oxalate is produced endogenously in the liver as the metabolic end product of various amino acids, ascorbate, glycine, and glyoxal (5, 22-24).

Oxalate is also found in the body after oxalate-containing foods are consumed. Most oxalate-containing foods are plants; common foods that are known to contain high levels of oxalate are spinach, rhubarb, chocolate, tea, beets, chard, nuts, and wheat bran (5). The reason why plants produce and store oxalate is still not completely known. It is speculated that the biosynthesis of oxalate in plants serves as a buffer in the tissues to counterbalance any cation-anion disproportions in the growing medium of the plant (25). It also appears that oxalate accumulation in the leaves of plants serves to resist pests and disease (26).

Oxalate may be in the form of insoluble or soluble salts. When oxalate is in the form of insoluble salts, the salts pass through the intestines to be excreted in the feces or degraded by oxalate-degrading bacteria known as *Oxalobacter formigenes*. However, when oxalate is in the form of soluble salt, it is believed to be readily absorbed in the digestive tract by passive diffusion in the small intestine and colon (5); or it may bind to divalent mineral cations such as calcium and magnesium, which causes the minerals to be unavailable for absorption and use by the body (5, 27, 28). Many studies have shown that urinary oxalate increases approximately one to six hours after ingestion of oxalate-
containing foods. This suggests that oxalate absorption in this time period occurs mostly in the small intestine of humans (29, 30).

Previously, dietary oxalate has been thought to account for only 10-15% of urinary oxalate; however, more recent research conducted by Holmes and colleagues (9) has shown dietary oxalate to contribute up to 50% of urinary oxalate. In a cross-sectional study using logistic regression conducted by Curhan and Taylor (31), they found that risk of stone formation increases with increases in urinary oxalate excretion (P<0.005). The study examined results from three large ongoing cohort studies: Nurses’ Health Study I (NHS I), Nurses’ Health Study II (NHS II), and Health Professionals Follow-up Study (HPFS). Table 1, adapted from this study, displays the multivariate relative risks for kidney stones according to category of 24-hr urinary concentration within NHS I, NHS II, and HPFS.

**Table 1. Multivariate relative risks for kidney stones according to category of 24-hr urinary oxalate excretion within NHS I, NHS II, and HPFS**

<table>
<thead>
<tr>
<th>Study</th>
<th>Oxalate (mg/L)</th>
<th>Cases</th>
<th>Controls</th>
<th>RR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;20</td>
<td>100</td>
<td>63</td>
<td>1.00 (Ref.)</td>
</tr>
<tr>
<td></td>
<td>20-24</td>
<td>171</td>
<td>105</td>
<td>1.15 (0.75-1.77)</td>
</tr>
<tr>
<td></td>
<td>25-29</td>
<td>224</td>
<td>99</td>
<td>1.59 (1.03-2.46)</td>
</tr>
<tr>
<td>NHS I</td>
<td>30-39</td>
<td>278</td>
<td>93</td>
<td>2.51 (1.59-3.96)</td>
</tr>
<tr>
<td>P, trend &lt;0.001</td>
<td>40+</td>
<td>125</td>
<td>43</td>
<td>2.36 (1.35-4.13)</td>
</tr>
<tr>
<td></td>
<td>&lt;20</td>
<td>116</td>
<td>75</td>
<td>1.00 (Ref.)</td>
</tr>
<tr>
<td></td>
<td>20-24</td>
<td>167</td>
<td>70</td>
<td>1.78 (1.14-2.80)</td>
</tr>
<tr>
<td></td>
<td>25-29</td>
<td>158</td>
<td>64</td>
<td>1.81 (1.13-2.90)</td>
</tr>
<tr>
<td></td>
<td>30-39</td>
<td>173</td>
<td>65</td>
<td>2.09 (1.27-3.43)</td>
</tr>
<tr>
<td>NHS II</td>
<td>40+</td>
<td>89</td>
<td>22</td>
<td>3.58 (1.85-6.94)</td>
</tr>
<tr>
<td>P, trend &lt;0.001</td>
<td>&lt;25</td>
<td>36</td>
<td>51</td>
<td>1.00 (Ref.)</td>
</tr>
<tr>
<td></td>
<td>25-29</td>
<td>72</td>
<td>45</td>
<td>2.35 (1.28-4.32)</td>
</tr>
<tr>
<td></td>
<td>30-39</td>
<td>203</td>
<td>146</td>
<td>1.90 (1.12-3.22)</td>
</tr>
<tr>
<td></td>
<td>40-49</td>
<td>178</td>
<td>92</td>
<td>2.67 (1.52-4.70)</td>
</tr>
<tr>
<td></td>
<td>50+</td>
<td>147</td>
<td>80</td>
<td>3.22 (1.72-6.05)</td>
</tr>
</tbody>
</table>

*RRs are adjusted for age, urinary creatinine, and all other urinary factors. Case and control numbers refer to the single 24-hr urine collection.

The large impact that dietary oxalate can have on urinary oxalate excretion is a testament as to why understanding the process and dynamics of oxalate metabolism is so important to reduce the prevalence and risk of recurrence of kidney stones. However, there are several factors that need to be considered that can cause variability in the available oxalate content of the foods. Some factors come into play prior to ingestion, while some do not have an effect until after digestion, absorption, and excretion of oxalate.

**Variability of Oxalate Content in Foods**

Oxalate contents of food can vary within plant species and even within a single plant. It is thought that oxalate can be transported from one part of the plant to others, depositing calcium and oxalate which may possibly be stored for later use by the plant. Within-species variability can be attributed to many growing conditions such as soil composition, amount of sunlight or other nutrients available during growth, geographic location, rate of growth, and timing of harvest (6, 7).

Typically, oxalate content is highest in the leaves, followed by seeds, and then stems. The content may change with the maturity of the plant and has been shown to increase as the plant ripens. In some cases, the accumulation of oxalate can even double during the aging process. This suggests that the end product of many metabolic pathways is oxalate. Conversely, some plants with a faster growth rate have been shown to have decreasing oxalate content with plant maturation, which possibly suggests that oxalate is needed for plant growth or seed production (5). Another study showed that young and mature beet leaves contained significantly less ($P<0.05$) total oxalate than regrowth and developed leaves, which also had the highest levels of soluble oxalate. Furthermore, the
developed leaves had significantly more (P<0.05) total oxalate than the regrowth. The researchers proposed that as leaves mature, oxalate is degraded or that increasing overall plant tissue results in lower oxalate content (32).

Bioavailability of Soluble Versus Insoluble Oxalate

As previously mentioned, oxalate can be either soluble or insoluble in foods. Normal oxalate absorption from foods has generally been found to be between 1% and 9% (33-37). One study conducted by Chai and Leibman (33) compared the absorption rates between almonds and black beans. They found that the absorption rate was higher in almonds (5.9%) than in black beans (1.8%). They attributed the differences to the relative amounts of soluble and insoluble oxalate in the foods since about 31% of oxalate in almonds is soluble, compared to only about 5% in black beans.

Bioaccessibility of Nutrients Within Food Matrices

The two forms of oxalate, soluble and insoluble, bring about questions as to the behavior of the two types of molecules once ingested. Insoluble (crystallized) oxalate is found within idioblasts of plants (8). Since insoluble oxalate is less likely to be absorbed due to the tight associations of the molecular constituents, we wonder what may happen if physical acts upon the idioblasts, such as chewing or blending prior to ingestion may render some oxalate more accessible for absorption.

Many studies have been conducted to better understand the effects of mastication on the bioaccessibility of different nutrients within food items. Bioaccessibility refers to the amount of nutrients within a food item that can be released from the food matrix. The
bioaccessibility of nutrients can be affected by the amount of mastication before being swallowed, or it can also be affected by food processing. For example, Lemmens and colleagues (38) tested the differences of bioaccessibility of β-carotene in raw, shredded carrots compared to cooked, pureed carrots. Patients with ileostomies were recruited to consume one of the two carrot preparations. After ingestion, stomal effluents were collected every two hours up to 12 hours. They found that the raw, shredded carrots remained physically intact with the only disruption of cells occurring at the surface of the shreds as a product of the initial grating. The cooked carrot contained free carotene-containing particles as well as intact cells in small clumps. Overall, they found that cells that had not been ruptured prior to ingestion during grating or pureeing remained intact and retained their carotenoid nutrients, rendering the nutrients unavailable. The only carotenoids that were made available for absorption were from cells which were ruptured prior to ingestion.

Another controlled dietary study investigating the bioaccessibility of β-carotene used differently processed spinach products to determine the role of cellular structure in digestion. There were four groups that received 20 gm of spinach. The spinach was whole leaf (almost intact food matrix), minced (partially disrupted matrix), enzymatically liquefied (disrupted matrix), or enzymatically liquefied to which dietary fiber was added. Significant differences were found among the groups in serum responses of β-carotene. Whole leaf spinach and minced spinach yielded significantly (P=0.03 and P=0.05, respectively) less serum responses than liquefied spinach. These results showed that the cellular integrity of spinach prior to digestion was an important factor in the bioaccessibility of β-carotene (39). Granted, β-carotene is hydrophobic and fat-soluble, unlike oxal-
late; it may behave differently from oxalate within idioblasts. However, we believe that comparable methods could be employed to better understand the bioaccessibility of oxalate within the food matrix of spinach.

Concomitant Foods

Many studies have shown that pairing of oxalate-containing foods with calcium- or magnesium-containing foods can affect the bioavailability and absorption of soluble oxalate. One study tested the effects of eating spinach (a high-oxalate food) with and without milk products (calcium-containing foods). They compared consumption of spinach alone or spinach with one of the following: sour cream, cottage cheese, sour cream plus Calci-Trim milk™, or olive oil. Oxalate content of the urine was measured after consumption at six hours and 24 hours. They found that consuming grilled spinach with sour cream and Calci-Trim milk™ significantly (P<0.05) reduced the availability of the oxalate in spinach. It is thought that the calcium in the dairy products binds to the soluble oxalate in the spinach. Pairing the spinach with fat did not influence the oxalate bioavailability, which suggests that fat content of a meal has no effect on the bioavailability of oxalate (40). These findings regarding the effect of dietary fat on oxalate absorption were contrary to the findings of Liebman et al (41) which showed fat as well as olestra depressed oxalate absorption in potatoes.

Effects of Calcium on Oxaluria

Previously, researchers have tried decreasing calcium in the diet in order to decrease hypercalciuria and stone risk. However, more recent studies have shown that while
decreasing dietary calcium reduces hypercalciuria, this reduction results in an increase in urinary oxalate and as a result, symptomatic kidney stones (14). When more calcium is present in the intestinal lumen, it forms a complex with oxalate and decreases oxalate absorption. However, with decreased calcium intake, less calcium in the lumen is present to bind with oxalate. Therefore, more oxalate is absorbed and excreted in the urine (19). In a study conducted by Holmes et al (9), normal subjects were on controlled diets of varying levels of oxalate and ratios of calcium to oxalate. Their results showed that oxalate excretion in the urine was significantly decreased by decreasing dietary oxalate and consuming normal amounts of calcium.

Another study showed that providing dietary and supplemental calcium to kidney stone formers reduced urinary oxalate excretion significantly (P=0.003 and P=0.038, respectively). The dietary calcium group received counseling to include at least 300 mg calcium with each meal, while the supplement group was counseled to consume calcium citrate supplements (300-500 mg) with meals as well as include dietary calcium with each meal. For both groups, the calcium oxalate supersaturation of the urine decreased significantly (P=0.043 and P=0.002, respectively). However, despite increases in ingested calcium, urinary calcium excretion did not differ significantly in either of the groups. Their data suggest that the binding of oxalate by calcium within the gastrointestinal tract renders the oxalate insoluble and therefore unabsorbed and that increasing daily calcium intake could be used as an effective strategy to decrease hyperoxaluria (42). Increasing daily calcium intake is now a common recommendation that clinicians make to patients to reduce urinary oxalate excretion, and hence, kidney stone risk.
Also of note, it has been demonstrated that increasing dietary oxalate can decrease the amount of calcium excreted in the urine (43). This is believed to happen because more dietary oxalate is available to bind with calcium in the lumen, rendering the calcium unavailable.

Effects of Food Processing on Oxalate Bioavailability

Chai and Liebman (44), among other researchers, have proven that boiling raw vegetables markedly reduces soluble oxalate content. They showed that boiling was more effective than steaming and baking and that after boiling the foods, the cooking water contained almost 100% of the oxalate lost in the foods. The amount of insoluble oxalate lost during boiling varied from 0 to 74%.

Simpson and colleagues (32) used the silver beet leaf (Beta vulgaris var. cicla) model to test the effect of cooking with different milk sources. The research showed that when leaves were cooked in milk, a significantly greater amount (P<0.05) of soluble oxalate was removed from the food when compared to cooking in water. Furthermore, they showed that the fat content of milk affected the soluble oxalate content as well, and low fat milk produced the largest decrease in oxalate content when compared to standard milk and cream.

Oxalobacter Formigenes

A certain bacterium, Oxalobacter formigenes, found in some humans has also been shown to play a role in the metabolism of oxalate in the gut (20). It is a gram-negative, anaerobic bacterium that can degrade oxalate utilizing a decarboxylase enzyme,
resulting in carbon dioxide and formate which is a less toxic acid than oxalic acid (45). Kaufman et al (46) used a case-control study to demonstrate that individuals who are colonized with these bacteria have a decreased risk of stone recurrence by 70%. In a study conducted by Jiang et al (43), participants who were colonized and not colonized with *O. formigenes* were placed on controlled diets with varying calcium and oxalate contents. Their findings suggested that colonization with *O. formigenes* decreases oxaluria when calcium intake is low and oxalate intake is moderate. When participants were on a low calcium/moderate oxalate diet, urinary oxalate excretion was reduced by 19.5% for colonized individuals when compared to non-colonized.

**Genetic Differences in Hyperoxaluria**

Another cause of hyperoxaluria is genetic variations. The disease is autosomal recessive, known as primary hyperoxaluria. So far, three types have been discovered. The most common and severe form is primary hyperoxaluria type 1 (PH1). It is characterized by underproduction of the liver peroxisomal alanine:glyoxylate aminotransferase (AGT) enzyme which is coded for by the AGXT gene. Upwards of 150 different mutations in the AGXT gene have been noted, with the majority being point mutations. The results are excessive endogenous oxalate production leading to urolithiasis, nephrocalcinosis, and progressively worsening renal function. Many of these patients eventually suffer from end-stage renal disease (ESRD) and eventually require dialysis or organ transplants (47).

Another genetic disease, known as primary hyperoxaluria type 2 (PH2), is also an autosomal recessive disorder. It too is characterized by increased urinary oxalate as well
as L-glycerate. The mutations are located in the GRHPR gene which codes for the glyoxylate:hydroxypyruvate reductase enzyme (48).

More recently, a third type of PH was determined in a cohort study of non-PH1/PH2 patients. The mutations were determined to be located in the HOGA1 gene on chromosome 10. This is now considered to be primary hyperoxaluria type 3 (PH3) (49).

Other Risk Factors of Hyperoxaluria

Increased urinary oxalate has also been shown to have an association with low levels of daily fluid intake, chronic low dietary calcium intake, increased animal protein consumption, increased sodium consumption, and increased dietary vitamin C (12-16). Adequate fluid intake is well-established to decrease the risk of stone formation through subsequent urine dilution (14, 16). Borghi et al (50) conducted a prospective controlled study that showed increasing daily fluid intake to produce a urine volume of 2.5 liters per day decreased stone recurrence. They documented that subjects who had experienced a kidney stone had significantly lower urine volumes than those who had not. For men, the mean difference was 344 mL of urine volume per 24 hours (P<0.0001); for women, the difference was 249 mL of urine volume per 24 hours (P<0.0001).

Despite the fact that hypercalciuria is a risk factor for kidney stones, dietary calcium has an inverse relationship with the risk of kidney stones (4, 14, 16, 51). As previously mentioned, lower calcium intake leads to increased oxalate excretion in the urine (9). Numerous studies have demonstrated the decreased stone risk with increased dietary calcium. This suggests that urinary oxalate levels may be more indicative of stone risk than urinary calcium levels, and increases in dietary calcium have only shown small increases
in urinary calcium (52). Also, decreased calcium intake could cause a negative calcium balance in the body and lead to withdrawal of calcium deposits from the bone which could result in decreased bone mineral density (this is seen mostly in patients with idiopathic hypercalciuria) (53).

Animal protein intake (not including dairy products) has been shown to increase excretions of uric acid (54) and calcium and lower urinary citrate excretion (55) (all factors to have a known predisposition for calcium stones). However, one study (56) showed that increasing animal protein while following a controlled oxalate diet did not have an effect on total oxalate urinary excretion. This data suggests that endogenous oxalate production is not affected by increased animal protein. So while increased animal protein has somewhat negative effects on 24-hour urine chemistries (57), no randomized clinical trial studies have definitively shown protein restriction to reduce stone occurrence. However, in a prospective study conducted by Curhan et al (14), food frequency questionnaires revealed that individuals who had increased animal protein intake had increased stone risk.

The fact that increased dietary sodium consumption increases urinary calcium excretion is well-established (13, 16). A cross-sectional study showed that individuals with the highest amount of urinary sodium had an increase of urinary calcium by 37 mg/day compared to individuals with the lowest sodium excretion (52). In a prospective cohort study with 12 years of follow-up, higher sodium in individuals’ diets was found to be positively associated with increased risk of kidney stones. Relative risk for women in the study who were in the group with highest average daily sodium intake was 1.30 (CI: 1.05-1.62) when compared to those in the group with lowest average daily sodium intake (53).
Another possible risk factor is the intake of vitamin C; through the metabolic pathways by which vitamin C is degraded, oxalate is a known end-product (56, 58, 59). One study found that 1.2 to 1.8% of ascorbic acid supplementation was converted to oxalate in vivo. This was the equivalent to increasing urinary oxalate from 6 to 13 mg in one day for each 1,000 mg of ascorbic acid provided, which could increase stone risk (60). However, the current tolerable upper limit for adults is 2,000 mg so many adults may be consuming well over 1,000 mg daily. In fact, one survey report showed that 67% of the U.S. population consumes vitamin supplements. Among the five most commonly used supplements were multivitamins containing vitamin C (71%) or vitamin C alone (32%), which commonly contain up to 1,000 mg per dose (61). Curhan et al (31) also showed that subjects with ascorbate intake of 1500 mg/day or greater had higher urinary oxalate levels.

Summary

All in all, it is fair to say that the entire process of oxalate production and metabolism is still not completely known since many variables effect the oxalate content in plants, bioaccessibility of that oxalate, and in vivo absorption. Our proposed research will hopefully provide additional insight to the bioavailability of dietary oxalate. The outcomes of this research have potential to provide patients and clinicians with knowledge to reduce kidney stone prevalence and recurrence.
METHODOLOGY

Subjects

In this crossover, controlled trial, healthy individuals of ages 19-45 years in close proximity to the University of Alabama at Birmingham (UAB) were recruited through advertisements on campus and word-of-mouth. Since many diseases and disorders can affect metabolism and excretion of nutrients, only healthy individuals were sought. Medical history and current health status was self-reported. Only individuals with a body mass index (BMI) of less than 30 kg/m$^2$ were included because higher BMIs could indicate metabolic disturbances. Due to the concurrent changes in nutrient metabolism, females were excluded if they were pregnant or lactating. Individuals with a known allergy or dislike for spinach were excluded. Age and ethnicity of the subjects were determined through self-report. All recruits were informed of the experimental design, and oral and written consent was obtained. The UAB Institutional Review Board approved the protocol (IRB # X140528013). We enrolled six healthy, non-stone forming, Caucasian individuals, three males and three females. Mean ± SD for participants’ age was 27.3 ± 3.3; BMI was 22.52 ± 2.20.

Protocol

There were three oxalate load treatment groups. Each of the six individuals participated in each of three loads. The three treatment loads were of differing sources/preparations of oxalate, and all three oxalate loads were served via smoothie. One
treatment served as a control group with soluble sodium oxalate salt being the source of oxalate in the smoothie; therefore, there was no spinach in the control smoothie. The other two treatment groups contained raw spinach, one containing raw spinach cut into 1 cm sq and the other containing raw, blended spinach.

Once enrolled, participants were instructed to refrain from a list of moderate to high oxalate foods for 24 hours prior to the oxalate load. Participants were asked to fast for at least twelve hours prior to the oxalate load and begin a 2-hr baseline urine collection on the morning of the scheduled load. They arrived at the UAB Clinical Research Unit (CRU), and upon completion of the urine collection participants received the oxalate load meal containing the oxalate smoothie. The smoothie consisted of 500 mg of oxalate in one of the three aforementioned forms. This breakfast meal also included a low-oxalate pastry and two cooked egg whites. This was a low-calcium meal in order to minimize the inhibitory effects of calcium on oxalate absorption. Participants then received instructions and materials for the remaining timed urine collections. They completed three additional 2-hr urine collections followed by a 16-hr urine collection to complete the 24-hr period. Participants returned the urine samples on the following day for oxalate content analysis. While at the CRU, participants also received a lunch and dinner meal to take with them for the remainder of the day. The meals for the remainder of the day contained a total less than 50 mg of oxalate and 150 mg of vitamin C. The lunch meal was also low in calcium, while the supper meal contained normal calcium (400-500 mg).

The total oxalate content of the diet is shown in Table 2. The dietary data were analyzed using Nutrition Data System for Research software version 2013, developed by the Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN.
**Table 2. Nutrient composition of study diet**

<table>
<thead>
<tr>
<th></th>
<th>Oxalate Load Smoothie</th>
<th>Breakfast*</th>
<th>Lunch</th>
<th>Dinner</th>
<th>Daily Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories (kcal)</td>
<td>242</td>
<td>246</td>
<td>825</td>
<td>522</td>
<td>1835</td>
</tr>
<tr>
<td>Oxalate (mg)</td>
<td>500</td>
<td>7</td>
<td>40</td>
<td>13</td>
<td>559</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>67</td>
<td>11</td>
<td>83</td>
<td>412</td>
<td>573</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>64</td>
<td>0</td>
<td>11</td>
<td>57</td>
<td>132</td>
</tr>
</tbody>
</table>

*Information for “breakfast” does not include nutrients from smoothie.

Measuring Urinary Ion Excretions

The method employed to measure oxalate bioavailability was previously described by Holmes et al (62) as the load method. This involves comparing the baseline amount of oxalate excreted for the two hours immediately before an oxalate load with oxalate excreted over 22 hours after the ingestion of an oxalate load. The amount of oxalate excreted after the load that is above the baseline period presumably represents the oxalate absorbed from the load. The amount of oxalate that is above the baseline period can be divided by the total amount of oxalate ingested to provide an estimate of oxalate absorption. This method assumes that the 2-hr baseline urinary oxalate is from endogenous oxalate synthesis; therefore, the individual must be fasted for at least ten hours prior to the beginning of the baseline urinary collection.

To measure the content of oxalate in urine, and therefore the amount of oxalate that was absorbed, we adopted a protocol utilizing a commercially available Oxalate Oxidase kit (Trinity Biotech). In addition, urinary creatinine was measured utilizing the EasyRA Chemistry Analyzer (Medica Corporation). Three urine aliquots were stored at -80°C: one untreated, one brought to pH 2, and one diluted 1:1 with HCl.

Variability of Oxalate Content in Spinach

To estimate the variation in the oxalate content of the spinach smoothies, we conducted oxalate analyses of spinach from four sources. Each sample was homogenized in
acid after being snap-freeze with liquid nitrogen and ground with a mortar and pestle to
determine the amounts of total oxalate. The oxalate measurements were conducted using
ion chromatography (IC) (Thermo Fisher Scientific, Inc, Waltham, MA).

Simulated in Vitro Digestion

The in vivo effects of oxalate digestion were compared to in vitro simulated di-
gestion. In order to do so, we employed methods similar to Muffarrij et al (63) and
Minekus et al (64). We prepared two buffers simulating the gastric and intestinal envi-
ronments. The oxalate sources included samples comparable to those in the in vitro ox-
alate loads – spinach completely blended and 1 cm sq spinach. The spinach was incubated
in a simulated gastric phase and intestinal phase, each for two hours. Supernatants from
each phase were obtained to be centrifuged, filtered, and diluted accordingly for oxalate
content analysis determined using IC. The amount of oxalate remaining in the superna-
tant was assumed to be the amount of oxalate extracted during the simulated digestion.
To estimate the percent of total oxalate extracted, the total oxalate concentration was
measured in spinach from the same source. The amount of oxalate extracted was then di-
vided by the total oxalate in the sample then multiplied by 100.

Statistical Considerations

The sample size is based primarily on feasibility considerations, such as resources
available to conduct the study, and is consistent with other recent studies in which urinary
oxalate excretion was measured (4, 9, 33, 40). However, the following power calculations
are provided in order to assess the adequacy of our sample size. All power calculations are performed using nQuery Advisor, version 7, and assume a two-sided statistical test, a type I error rate of 0.05, and a sample size of 6 participants. Using information from Brogren and Savage (40), we obtained estimates of the standard deviation for the mean urinary oxalate of 3.7 mg/6 hrs and 7.4 mg/24 hrs. With these assumptions, and that of a paired t test, we will have 80% power to detect a significant change in the urinary oxalate content between baseline and 6 hrs of 5.4 mg/6 hrs (or greater), and between baseline and 24 hrs of 10.7 mg/24 hrs (or greater).

Descriptive statistics, such as means and standard deviations, were used to summarize demographic and clinical characteristics, and quantitative measures, including urinary volume, creatinine, oxalate, oxalate(mg)/creatinine(g) ratio (Ox/Cr), and percent bioavailability (%BA). Distributions of urinary study variables were examined using stem-and-leaf plots, normal probability plots, and the Kolmogorov-Smirnov test, and it was determined that the distributions did not deviate greatly from a normal distribution. The primary method of statistical analysis was mixed models repeated measures analyses (i.e., repeated measures analysis of covariance where the covariance matrix can be selected). A compound symmetry covariance matrix was used for these models since we expected to see constant correlation among the changes for each participant (each visit was completed in 24 hours and only a single visit took place for each smoothie). The Tukey-Kramer multiple comparisons test was then used to determine which pairs of means were significantly different. These models included terms for group, time, and group by time interaction. Mixed models repeated measures analyses were also used to perform comparisons between groups at baseline (only for urinary volume, creatinine, oxalate, and oxal-
late/creatinine ratio) and between groups for the sum of the 2, 4, and 6 hour measurements (for all 5 urinary variables). Analysis of variance was used for the comparison of the four groups (gastric and intestinal) for the in vitro data, with the Tukey-Kramer test being the multiple comparisons test. Statistical tests were two-sided. Values of $P < 0.05$ were considered to designate statistical significance. Statistical analyses were performed using SAS (version 9.4; SAS Institute, Inc., Cary, NC).
RESULTS

Urinary Measurements

The amount of oxalate that was absorbed after the ingestion of spinach and excreted in urine was calculated using two different methods. The first method involved subtracting the 2-hr baseline urinary oxalate measurement from each post-load urinary oxalate value within one 24-hr period, assuming this amount to be endogenous oxalate production. The second method to assess results determined the ratio of oxalate (mg) to the creatinine (g) clearance for the same specified time period. Tables 3-6 list baseline and post-load urine volume, creatinine, Ox/Cr values for each oxalate load (N=6 for each group). Post-load oxalate values are corrected for the baseline, presumably endogenous, oxalate values. Since previous research has shown peak urinary oxalate excretion to occur mainly six hours after ingestion of oxalate, the data have been analyzed at individual urine time points across the 24-hr period as well as a sum of the first six hours and as a total 24-hr sum.

Table 3. Mean ± SD baseline and post-load urinary volume (mL)

<table>
<thead>
<tr>
<th>Oxalate Load (N =6)</th>
<th>Blended</th>
<th>1 cm sq</th>
<th>Sodium Oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>146 ± 107</td>
<td>118 ± 36</td>
<td>131 ± 44</td>
</tr>
<tr>
<td>2-hr</td>
<td>553 ± 176</td>
<td>642 ± 242</td>
<td>404 ± 247</td>
</tr>
<tr>
<td>4-hr</td>
<td>453 ± 101</td>
<td>489 ± 182</td>
<td>533 ± 128</td>
</tr>
<tr>
<td>6-hr</td>
<td>317 ± 90</td>
<td>389 ± 208</td>
<td>337 ± 137</td>
</tr>
<tr>
<td>24-hr</td>
<td>1263 ± 361</td>
<td>915 ± 388</td>
<td>1016 ± 410</td>
</tr>
<tr>
<td>Total 24-hr</td>
<td>2732 ± 394</td>
<td>2553 ± 492</td>
<td>2421 ± 474</td>
</tr>
<tr>
<td>Sum of 1st 6 hrs</td>
<td>1323 ± 142</td>
<td>1520 ± 410</td>
<td>1274 ± 329</td>
</tr>
</tbody>
</table>
Table 4. Mean ± SD baseline and post-load urinary creatinine (mg)

<table>
<thead>
<tr>
<th>Oxalate Load</th>
<th>Baseline</th>
<th>1 cm sq</th>
<th>Sodium Oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blended</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>145.9 ± 76.1</td>
<td>108.4 ± 36.0</td>
<td>152.1 ± 54.0</td>
<td></td>
</tr>
<tr>
<td>2-hour</td>
<td>140.3 ± 59.0</td>
<td>125.0 ± 46.5</td>
<td>152.1 ± 64.9</td>
</tr>
<tr>
<td>4-hour</td>
<td>143.9 ± 42.8</td>
<td>128.6 ± 37.8</td>
<td>119.2 ± 28.7</td>
</tr>
<tr>
<td>6-hour</td>
<td>163.6 ± 65.1</td>
<td>135.6 ± 30.4</td>
<td>133.3 ± 65.3</td>
</tr>
<tr>
<td>24-hour</td>
<td>1223.1 ± 359.8</td>
<td>1137.8 ± 417.8</td>
<td>1082.3 ± 405.7</td>
</tr>
<tr>
<td>Total 24-hour</td>
<td>1816.8 ± 584.1</td>
<td>1635.3 ± 515.9</td>
<td>1639.0 ± 602.6</td>
</tr>
<tr>
<td>Sum of 1st 6 hrs</td>
<td>447.8 ± 163.4</td>
<td>389.2 ± 106.7</td>
<td>404.6 ± 157.0</td>
</tr>
</tbody>
</table>

Table 5. Mean ± SD baseline and post-load urinary oxalate (mg)

<table>
<thead>
<tr>
<th>Oxalate Load</th>
<th>Baseline</th>
<th>1 cm sq</th>
<th>Sodium Oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blended</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.24 ± 0.47a</td>
<td>1.40 ± 0.37ab</td>
<td>2.11 ± 0.76b</td>
<td></td>
</tr>
<tr>
<td>2-hour</td>
<td>3.10 ± 0.85</td>
<td>2.49 ± 0.85</td>
<td>5.19 ± 2.63</td>
</tr>
<tr>
<td>4-hour</td>
<td>6.39 ± 1.89</td>
<td>7.42 ± 3.11</td>
<td>9.65 ± 2.64</td>
</tr>
<tr>
<td>6-hour</td>
<td>5.24 ± 1.99</td>
<td>6.65 ± 2.56</td>
<td>6.90 ± 5.12</td>
</tr>
<tr>
<td>24-hour</td>
<td>11.60 ± 6.05</td>
<td>14.22 ± 4.55</td>
<td>13.18 ± 15.80</td>
</tr>
<tr>
<td>Total 24-hour</td>
<td>26.33 ± 8.01</td>
<td>30.78 ± 9.14</td>
<td>34.92 ± 23.12</td>
</tr>
<tr>
<td>Sum of 1st 6 hrs</td>
<td>14.73 ± 3.54a</td>
<td>16.56 ± 5.27ab</td>
<td>21.74 ± 8.63b</td>
</tr>
</tbody>
</table>

*Post-load values are adjusted for baseline measurements
Values within a row with different superscript letters are significantly different (P<0.05)

Table 6. Mean ± SD baseline and post-load urinary Ox(mg)/Cr(g)

<table>
<thead>
<tr>
<th>Oxalate Load</th>
<th>Baseline</th>
<th>1 cm sq</th>
<th>Sodium Oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blended</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.52 ± 6.44</td>
<td>13.25 ± 2.10</td>
<td>15.18 ± 7.03</td>
<td></td>
</tr>
<tr>
<td>2-hour</td>
<td>23.60 ± 6.04</td>
<td>20.73 ± 7.34</td>
<td>36.21 ± 21.56</td>
</tr>
<tr>
<td>4-hour</td>
<td>46.56 ± 16.45a</td>
<td>55.62 ± 15.34a</td>
<td>84.45 ± 29.32b</td>
</tr>
<tr>
<td>6-hour</td>
<td>35.42 ± 18.13</td>
<td>48.89 ± 15.68</td>
<td>52.37 ± 24.17</td>
</tr>
<tr>
<td>24-hour</td>
<td>10.71 ± 6.98</td>
<td>13.24 ± 5.56</td>
<td>12.21 ± 14.43</td>
</tr>
<tr>
<td>Total 24-hour</td>
<td>16.00 ± 7.62</td>
<td>19.25 ± 5.29</td>
<td>21.81 ± 13.02</td>
</tr>
<tr>
<td>Cumulative 1st 6 hrs</td>
<td>35.35 ± 12.38a</td>
<td>42.23 ± 6.02ab†</td>
<td>56.64 ± 21.62b†</td>
</tr>
</tbody>
</table>

*Post-load values are adjusted for baseline measurements
† While the differences between the 1cm sq and sodium oxalate loads are not significantly different, there is a trend toward significance (P=0.069)
Values within a row with different superscript letters are significantly different (P<0.05)

Analysis of baseline measures showed no significant differences among the three groups for volume, creatinine or Ox/Cr. There was a significant difference for baseline urinary oxalate among the three groups (P=0.032); the mean of the sodium oxalate
smoothie was significantly greater than the mean for the blended smoothie (P=0.035). Longitudinal analysis of the three groups and the five time points (baseline, 2-hr, 4-hr, 6-hr, and 24-hr) showed no significant differences in group or group by time interactions for volume, creatinine, or oxalate. The differences for time were significant for each measure (P<0.001 for all). The longitudinal analysis for Ox/Cr showed that the differences among groups were significant (P=0.004), and the group by time interaction was significant (P=0.044) The mean of Ox/Cr for the sodium oxalate load is significantly greater than the blended spinach load at 4-hr (P=0.002) and significantly greater than the 1 cm sq spinach load at 4-hr (P=0.036). These results are expressed in Figure 1.

**Figure 1. Comparison of Urinary Ox(g)/Cr(mg)**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Ox (mg)/Cr (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

The %BA index was estimated by dividing the urinary oxalate value by the amount of oxalate provided in the load. These results are listed in Table 7 and expressed in Figure 2.
Table 7. Mean ± SD Percent Bioavailability of Oxalate

<table>
<thead>
<tr>
<th>Oxalate Load</th>
<th>Blended</th>
<th>1 cm sq</th>
<th>Sodium Oxalate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
<td>0.00 ± 0.0</td>
</tr>
<tr>
<td>2-hr</td>
<td>0.62 ± 0.2</td>
<td>0.50 ± 0.2</td>
<td>1.04 ± 0.5</td>
</tr>
<tr>
<td>4-hr</td>
<td>1.28 ± 0.4</td>
<td>1.49 ± 0.6</td>
<td>1.93 ± 0.5</td>
</tr>
<tr>
<td>6-hr</td>
<td>1.05 ± 0.4</td>
<td>1.33 ± 0.5</td>
<td>1.38 ± 1.0</td>
</tr>
<tr>
<td>24-hr</td>
<td>2.32 ± 1.2</td>
<td>2.85 ± 0.9</td>
<td>2.64 ± 3.2</td>
</tr>
<tr>
<td>Total 24-hr</td>
<td>5.27 ± 1.6</td>
<td>6.16 ± 1.8</td>
<td>6.99 ± 4.6</td>
</tr>
</tbody>
</table>

Sum of 1st 6 hrs | 2.95 ± 0.71<sup>a</sup> | 3.32 ± 1.05<sup>ab</sup> | 4.35 ± 1.73<sup>b</sup> |

Values within a row with different superscript letters are significantly different (P<0.05)

Figure 2. Comparison of Cumulative Percent Bioavailability of Oxalate

Longitudinal analysis of the three groups and five time points for the %BA showed no significant group differences or group by time interactions; the time differences were significant (P<0.001).

While group differences were present for Ox/Cr across the 24-hr period, mean total 24-hr values for oxalate excretion, Ox/Cr, and %BA among the groups were not significantly different. However at four hours post-load, the oxalate values peaked and the differences were most pronounced. For the sum of the first six hours post-load, urinary
oxalate, Ox/Cr, and %BA were all significantly different among the three group means (P=0.025, P=0.011, P=0.026, respectively). The values were highest for the sodium oxalate load, followed by the 1cm sq spinach, and then the blended spinach. By 24 hours post-load, urinary oxalate values normalized and values were similar among the groups for each of the four oxalate measurements.

Of the four participants that were tested for *Oxalobacter formigenes* colonization; all were negative.

**Variability of Oxalate Content in Spinach**

As previously stated, oxalate content of spinach can be highly variable based on many factors. In order to gain some insight to the degree of variability to expect, the oxalate content of four different spinach samples was measured. These were samples which were randomly selected from spinach that was fed to participants in the in vivo portion of the experiment. The oxalate content among the spinach samples had a mean (± SD) of 851.62 ± 155.20 mg oxalate/100gm spinach and the means were significantly different (P=0.008). These results were near expected values and similar to previous reports.

**In Vitro Simulated Digestion**

The results from the in vitro experiments are summarized in Table 8. Each of the experiments was performed in quadruplicate.
Table 8. Percent of Total Oxalate Extracted (mean ± SD) During in vitro Simulated Digestion

<table>
<thead>
<tr>
<th>Spinach sample</th>
<th>Gastric Phase (N=4)</th>
<th>Intestinal Phase (N=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blended</td>
<td>68.09 ± 5.39</td>
<td>79.96 ± 6.51</td>
</tr>
<tr>
<td>1 cm sq</td>
<td>22.07 ± 1.91</td>
<td>92.35 ± 4.96</td>
</tr>
</tbody>
</table>

*all results were significantly different from one another (P<0.001)

The mean amount of oxalate extracted (±SD) from blended spinach after the gastric phase was 68.09% (± 5.39) and after the intestinal phase was 79.96% (± 6.51). The blended spinach gastric phase extracted significantly less oxalate than the final amount extracted after the intestinal phase (P=0.025). The mean amount of oxalate extracted from the 1cm sq gastric phase spinach was 22.07% (± 1.91) and after the intestinal phase was 92.35% (± 4.96). The gastric phase of 1cm sq spinach extracted significantly less oxalate than the intestinal phase (P<0.001). All of the means of the four groups are significantly different from one another (P<0.001). The blended spinach group resulted in significantly less total oxalate extracted than the 1cm sq group (P=0.02).
DISCUSSION

Urinary Oxalate

The only significant difference among the groups at baseline was in urinary oxalate excretion. Since urinary volume, creatinine, and Ox/Cr were the same across groups at baseline, there was no need to control for any of these variables. Differences in urinary oxalate values at baseline could be indicative of variability of endogenous oxalate production rates within individuals, differences in self-selected diets for the days leading up to the oxalate load, or error in timing of urine collections since Ox/Cr was not different among the groups. Since a minimum wash-out period of one week was in place between the oxalate loads, the urinary oxalate differences at baseline among the groups are not attributed to a remaining effect from a previous oxalate load.

These results demonstrate that while there were no significant differences in the three 24-hr urinary oxalate measurements (oxalate, Ox/Cr, and %BA) among the groups, the mean of each measurement was significantly different for the sum of the first six hours following the oxalate loads. The sodium oxalate load had highest measured bioavailability which was in accordance with our hypothesis; there were no significant differences between the means of the blended spinach load and the 1 cm sq spinach load.

The 24-hr %BA of the sodium oxalate load is comparable to previous reports of a similar load. In a study conducted by Knight, Holmes, and Assimos, (65) the percent bioavailability for a sodium oxalate load was around 8%. We found that number to be
around 7%, but that small difference could be attributed to the differences between the two studies in oxalate and calcium content in the oxalate load and meals for the remainder of the day given to the participants.

There were no significant differences between the blended spinach load and the 1 cm sq spinach load; while this is different from expected, there may be several reasons to explain why no differences were present. The 24-hr period we observed may not be a sufficient amount of time to measure total oxalate excretion. A previous study found that, taking into account the activity in the gut, some of the dietary oxalate can take four to five full days to be excreted (62). Due to study feasibilities, we had a small sample size of six individuals. If we were to test our hypothesis in a larger group, perhaps there would be enough data to reveal statistically significant differences. In addition, the hypothesis was tested in healthy individuals with normal renal and intestinal function. Results may be different for individuals with hyperoxaluria or other predispositions for kidney stones, such as overt intestinal disease.

Since each of the four participants that were tested for colonization with Oxalobacter formigenes were negative, we do not attribute colonization status to be a crucial factor influencing the variability we found in absorption rates among participants. Although the entire gut microflora may play a role, to this date this has not been researched.

In Vitro Simulated Digestion

The 1 cm sq spinach yielded significantly higher oxalate extraction than the blended spinach (P=0.02) in the simulated digestion experiment; this could suggest that
the particle size of spinach does affect the way that oxalate behaves on a molecular level after ingestion and that blending spinach has some effect that inhibits the amount of oxalate that is available for absorption. In the in vivo experiment, the mean values for the 1 cm sq spinach were slightly greater, although not significantly, than the values for the blended spinach. This in vitro experiment suggests that perhaps in a more controlled in vivo experiment or larger sample size, the 1 cm sq spinach load would result in significantly higher oxalate absorption than the blended spinach load.

Variability of Oxalate Content in Spinach

The variability of oxalate content in the spinach used in the smoothies may have slightly impacted the urinary oxalate results. One way to control for this variability would be to freeze one batch of spinach from the same source and use that for all twelve spinach smoothies. However, the requirement to use fresh spinach was important as the freeze-thaw process could influence the behavior of the oxalate in the plant cells. Another possible solution to this could be measuring the oxalate content of each spinach source used and adjusting the amount of spinach used in the smoothie to equal exactly 500mg of oxalate. However, due to the inability to obtain rapid oxalate measurements, this method would not be feasible. Furthermore, adjusting the amount of spinach used would change the composition of the smoothies which could lead to further unknown confounding variables. For these reasons, we elected to use fresh spinach for the spinach oxalate loads. Additionally, when the results using the value of the actual versus the average oxalate content of the spinach were compared, no significant differences were found.
Study Limitations and Strengths

As with most studies, we do recognize some limitations. Since the first urine collection is considered the endogenous, baseline urinary oxalate production for participants, it is pertinent that they follow a low-oxalate diet prior to participation. Providing meals to participants for days leading up to participation would be the most ideal way to control this variable. However, providing individuals a controlled diet was not economically feasible for this project. For this reason, we provided participants with a list of high oxalate foods to avoid for 24 hours prior to participation.

Another limitation is the degree to which we rely on the participants to accurately follow the controlled diet and to accurately collect the timed urine samples. At any micturition, unless the individual is catheterized, it is nearly impossible to empty absolutely all of the urine from the bladder. On average a person could leave 20 to 30 mL of urine in the bladder which could affect the results we measured. Since the results for the differences among the groups were more significant when measures of urinary oxalate were related to creatinine than when not related to creatinine, this could indicate that errors in urine collections could skew the results and hide significance.

Most of the studies previously conducted to estimate bioavailability of nutrients within food observe in vitro models. While these studies can serve as a cost-effective and safe way to assess nutrient kinetics, to this date there is no way to completely mimic the entire biological and physiological systems that happen in vitro. Of the in vitro study models conducted in the past assessing nutrient bioavailability, almost all studies employ
the use of frozen foods. While this does ensure uniformity among participant treatments, freezing and thawing a food can affect how the food behaves once it is ingested. Our current research is novel in that we have used techniques that are often avoided due to uncertainty of outcomes or unknown variables.

Implications of the Study

This study implies that the maceration and intestinal digestion of spinach does not significantly affect bioavailability of oxalate in healthy individuals over a 24-hr period, but it may influence the rate of absorption in the hours immediately following ingestion. However, one important factor to consider is that we tested our hypothesis in healthy individuals who reported no history of kidney stones, renal function decline, or factors influencing gut health or nutrient absorption and/or metabolism. The overarching goal of this study is to decrease the prevalence of kidney stones in individuals with a predisposition for urolithiasis. The results may prove to be different or have different implications if this study were conducted in a diseased population.

Previous case studies have shown oxalate nephropathy, acute renal failure, and even death due to juicing of high oxalate foods in patients with chronic kidney disease or overt intestinal disease (66, 67). This is one reason we decided to test how the mechanical phase of a high-oxalate food may influence nutrient bioavailability. While increasing dietary oxalate is certainly a risk for urolithiasis here, there are multiple other risks to juicing and blending foods. Beyond the increase in dietary oxalate consumption, juices are usually low in calcium, high in vitamin C, and increase the individual’s fluid intake dramati-
cally. The high fluid amounts could cause some of the calcium oxalate to remain soluble and more oxalate to be absorbed (66). All of these are risk factors beyond the oxalate content and/or form of foods for urolithiasis, especially within a population with a predisposition for hyperoxaluria.

Conclusions

While our research did not show statistically significant differences in 24-hr urinary oxalate excretion after ingestion of dietary spinach oxalate loads with varying degrees of homogenization, some trends did arise that suggest that mechanical phase of spinach can affect the timing and kinetics of oxalate absorption for the hours immediately following ingestion. It is possible that if this hypothesis was tested within a population with predisposition for hyperoxaluria or overt intestinal disease, the bioavailability differences would become statistically significant.
LIST OF REFERENCES


47. Mandrile G, van Woerden CS, Berchialla P, Beck BB, Acquaviva Bourdain C, Hulton SA, Rumsby G. Data from a large European study indicate that the outcome of primary hyperoxaluria type 1 correlates with the AGXT mutation type. Kidney Int 2014. doi: 10.1038/ki.2014.222. [Epub ahead of print]


49. Belostotsky R, Seboun E, Idelson GH, Milliner DS, Becker-Cohen R, Rinat C, Monica CG, Feinstein S, Ben-Shalom E, Magen D, et al. Mutations in DHDPSL are re-


APPENDIX

INSTITUTIONAL REVIEW BOARD APPROVAL
UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on January 24, 2017. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

Principal Investigator: HOLMES, ROSS P.
Co-Investigator(s): KNIGHT, JOHN
MITCHEM, APRIL L. ELLIS

Protocol Number: X140528013
Protocol Title: Bioavailability of Dietary Oxalate

The IRB reviewed and approved the above named project on 5-12-14. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received EXPEDITED review.

IRB Approval Date: 5-12-14
Date IRB Approval Issued: 5-12-14
IRB Approval No Longer Valid On: 6-12-15

Marilyn Doss, M.A.
Vice Chair of the Institutional Review Board for Human Use (IRB)

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

470 Administration Building
701 20th Street South
205.934.3789
Fax 205.934.1301
irb@uab.edu

The University of Alabama at Birmingham
Mailing Address:
AB 470
1720 2ND AVE S
BIRMINGHAM AL 35294-0104
CONSENT FORM

TITLE OF RESEARCH:  Bioavailability of Dietary Oxalate

IRB PROTOCOL:  X140528013

INVESTIGATOR:  Ross P. Holmes, Ph.D.

SPONSOR:  UAB Department of Urology

Purpose of the Research

We are asking you to take part in a research study. Please read this information carefully and feel free to ask questions about anything you do not understand. This research study will test how the method of preparing spinach (i.e., raw, heated, blended, minimally chewed) effects how well the body absorbs a nutrient known as oxalate.

Oxalate is a small molecule often found in leafy vegetables and grains. Much of the oxalate found in the body is excreted in the urine and can cause a problem for individuals with kidney stone disease when it forms crystals with calcium. People who enter into the study will consume spinach with one or more methods of preparation.

There are four different spinach preparations. We plan to have at least six individuals consume each spinach preparation. We expect to enroll approximately 24 healthy adult men and women.

Explanation of Procedures

If you enter the study, you will be asked to complete at least one of the four spinach preparations; you may participate in all four if you choose to do so. For one spinach preparation you will be asked to do the following:

1. Empty your bladder on the morning of your appointment and begin a 2-hour urine collection. Before the two hours are completed, you will be asked to come to the UAB Clinical Research Unit (CRU).
2. Once at the CRU after the 2-hour urine collection is completed, you will be asked to consume a breakfast containing spinach.
3. Following breakfast, you will continue to complete three 2-hour urine collections. We will provide a lunch and dinner for you this day as well.
4. When the 2-hour urine collections are finished, you will complete a 16-hour urine collection (to complete a 24-hour collection).
Each timed urine collection will require you to collect all of the urine your body produces for the designated amount of time (either two hours or 16 hours) in the same container.

5. You will be asked to return the urine samples upon completion of the 24 hours. If you enter the study, you will be asked to refrain from any vitamins or supplements that can effect nutrient excretion in the urine.

Risks and Discomforts

There is a slight risk that your private health information may be exposed. However, precautions will be taken to limit any such exposure. There is also a slight risk that food may be improperly prepared. Persons who consume food that is improperly prepared may experience nausea, vomiting, or diarrhea. However, the CRU Metabolic Kitchen follows correct policies and procedures to ensure food safety. There are no risks associated with obtaining urine samples.

Benefits

You will not benefit directly from taking part in this study. However, by participating in the study you will help us understand the factors associated with the bioavailability of oxalate.

Alternatives

This is not a treatment study; your participation is completely voluntary. Your alternative is to not participate in this research study.

Confidentiality

Information obtained about you for this study will be kept confidential to the extent allowed by law. However, research information that identifies you may be shared with the UAB Institutional Review Board (IRB) and others who are responsible for ensuring compliance with laws and regulations related to research, including people on behalf of the Office for Human Research Protections (OHRP). The results of the treatment may be published for scientific purposes. These results could include your lab tests. However, your identity will not be given out.

Voluntary Participation and Withdrawal

Whether or not you take part in this study is your choice. There will be no penalty if you decide not to be in the study. If you decide not to be in the study, you will not lose any benefits you are otherwise owed. You are free to withdraw from this research study at any time. Your choice to leave the study will not affect your relationship with this institution.
You may be removed from the study without your consent if the sponsor ends the study or if the study doctor decides it is not in the best interest of your health, or if you are not following the study rules.

If you are a UAB student or employee, taking part in this research is not a part of your UAB class work or duties. You can refuse to enroll, or withdraw after enrolling at any time before the study is over, with no effect on your class standing, grades, or job at UAB. You will not be offered or receive any special consideration if you take part in this research.

Cost of Participation

There will be no cost to you for taking part in this study.

Payment for Participation in Research

You will be paid $50 for each completed visit including the 24 hours of urine collection. If you quit the study, you will be paid $7.50 for each sample that you have provided. If you participate in and complete all four study visits, you may receive up to $200. Payments will be made by check sent to you in the mail. You may have the opportunity to complete up to four study visits.

Payment for Research-Related Injuries

UAB has not provided for any payment if you are harmed as a result of taking part in this study. If such harm occurs, treatment will be provided. However, this treatment will not be provided free of charge.

Significant New Findings

You will be told by your doctor or the study staff if new information becomes available that might affect your choice to stay in the study.

Questions

If you have any questions, concerns, or complaints about the research or a research-related injury including available treatments, you may contact Dr. Ross Holmes. He will be glad to answer any of your questions. Dr. Holmes' number is 205-996-2291. Dr. Holmes may also be reached after hours at 334-831-7821.

If you have questions about your rights as a research participant, or concerns or complaints about the research, you may contact the UAB Office of the IRB (OIRB) at (205) 934-3789 or toll free at 1-855-860-3789. Regular hours for the OIRB are 8:00 a.m. to 5:00 p.m. CT, Monday through Friday. You may also call this number in the event the research staff cannot be reached or you wish to talk to someone else.

Page 3 of 5
Version Date: 6/6/2014
Legal Rights

You are not waiving any of your legal rights by signing this informed consent document.

Signatures

Your signature below indicates that you agree to participate in this study. You will receive a copy of this signed document.

Signature of Participant

Signature of Principal Investigator

Signature of Witness

Date

Date

Date
What is the purpose of this form? You are being asked to sign this form so that UAB may use and release your health information for research. Participation in research is voluntary. If you choose to participate in the research, you must sign this form so that your health information may be used for the research.

**Participant Name:** 
**Research Protocol:** Bioavailability of Dietary Oxalate  
**UAB IRB Protocol Number:** X140528013  
**Principal Investigator:** Ross P. Holmes, Ph.D.  
**Sponsor:** UAB Department of Urology

What health information do the researchers want to use? All medical information and personal identifiers, including past, present, and future history, examinations, laboratory results, imaging studies and reports and treatments of whatever kind related to or collected for use in the research protocol.

Why do the researchers want my health information? The researchers want to use your health information as part of the research protocol listed above and described to you in the Informed Consent document.

Who will disclose, use and/or receive my health information? The physicians, nurses and staff working on the research protocol (whether at UAB or elsewhere); other operating units of UAB, HSF, UAB Highlands, Children’s of Alabama, Eye Foundation Hospital and the Jefferson County Department of Public Health, as necessary for their operations; the IRB and its staff; the sponsor of the research and its employees; and outside regulatory agencies, such as the Food and Drug Administration.

How will my health information be protected once it is given to others? Your health information that is given to the study sponsor will remain private to the extent possible, even though the study sponsor is not required to follow federal privacy laws. However, once your information is given to other organizations that are not required to follow federal privacy laws, we cannot assure that the information will remain protected.

How long will this Authorization last? Your authorization for the uses and disclosures described in this Authorization does not have an expiration date.

Can I cancel the Authorization? You may cancel this Authorization at any time by notifying the Director of the IRB, in writing, referencing the Research Protocol and IRB Protocol Number. If you cancel this Authorization, the study doctor and staff will not use any new health information for research. However, researchers may continue to use the health information that was provided before you cancelled your authorization.

Can I see my health information? You have a right to request to see your health information. However, to ensure the scientific integrity of the research, you will not be able to review the research information until after the research protocol has been completed.

Signature of participant: ___________________________ Date: __________  
or participant’s legally authorized representative: ___________________________ Date: __________  
Printed Name of participant’s representative: ___________________________  
Relationship to the participant: ___________________________
MEMORANDUM

Date: March 4, 2015

From: Misty L. White, MA
  Protocol Analyst II

To: Mary C. Robertson

Re: X140528013

This memorandum is to confirm that Mary C. Robertson is listed as a Co-Investigator for UAB IRB Protocol Number X140528013: Bioavailability of Dietary Oxalate. Dr. Ross Holmes is the Principal Investigator of the study.