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Effects of Grain Sorghum Muffin on Blood Glucose and Insulin Responses in Prediabetic Men

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Citation

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Abstract

Prediabetes is a sub-health condition in the development to type 2 diabetes, which has been long overlooked. Grain sorghum contains functional starch fractions, which have been widely reported for their potential on blood glucose control and diabetes prevention. A human study with prediabetic men was conducted to investigate the effects of sorghum starch on postprandial blood glucose and insulin levels. Grain sorghum and wheat (control) muffins containing 50 g total starch were consumed by 15 prediabetic male subjects on two mornings with a 1-week washout period. Plasma samples were collected on -15 (baseline), 0, 15, 30, 45, 60, 75, 90, 120, and 180 minutes after each treatments. The functional starch content (SDS and RS combined) of grain sorghum muffin was higher than control muffin. Postprandial blood glucose and insulin responses were both significantly reduced on 45 – 120 min intervals ($p<0.05$). With the grain sorghum muffin treatment, the mean incremental area under the curve (iAUC) of glucose was significantly reduced by 35.0%, from 5457.5 ± 645.4 to 3550.0 ± 428.9 mg (~3 h) dL^{-1} ($P<0.05$). The mean iAUC of insulin was also significantly lowered by 36.7%, from 7254.6 ± 1228.9 to 4589.3 ± 737.2 mU (~3 h) L^{-1} ($p<0.05$). The results implied that grain sorghum is a good candidate in controlling blood glucose and insulin levels in prediabetic population for the prevention of type 2 diabetes.

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Introduction

According to CDC diabetes report[1], 29.1 million or 9.3% of the US population are diagnosed with diabetes mellitus. Prediabetes is a health condition between normal and diabetes, characterized by a fasting plasma glucose level between 100 and 125 mg/dL. Prediabetic people have higher risk of type 2 diabetes compared with normal population. According to CDC's estimation[2], 15 – 30% of the prediabetic people can develop to type 2 diabetes in 3-5 years. In addition, CDC's report shows that only few prediabetic people are aware of their condition – <14% by estimation[2].

Grain sorghum is world's fifth most important cereal crop[3,4]. Sorghum is an important animal feed used in the US and white sorghum product is used to a small extent to substitute for wheat. Grain sorghum is very similar as other crop grains, composed of pericarp, testa, endosperm and embryo, and total starch content can range from 55 to 76% depending on crop and cultivar[5,6]. The indigestible properties of grain sorghum have been recognized[6-9]. These properties would be associated with health benefits such as lower blood sugar, decreased insulin release, increased satiety, and weight control.

The **goal** of this study is to examine the effects of functional starch in grain sorghum, which may be used for the prevention of diabetes. The **hypothesis** of this study is that grain sorghum muffin will reduce postprandial blood glucose and insulin responses in people with prediabetes. The **objectives** of this study are to measure functional starch contents in grain sorghum and to assess blood glucose and insulin responses after consumption of grain sorghum muffin in prediabetic men.

Literature Review

National Diabetic/Prediabetic Facts

In the National Diabetes Statistics Report 2014 [1], 29.1 million people or 9.3% of the population in the United States are estimated to have diabetes. Among those, 21 million people are already diagnosed with diabetes and 8.1 million people are undiagnosed[1]. The diabetic population and percentage has dramatically increased in the last several decades (Figure 1). As illustrated in Figure 1, the diagnosed diabetic population in 1958 was only 1.58 million (0.93%) and diabetic population was growing steadily to 7.63 million (2.89%) in 1996. The diagnosed diabetes population then steeply increased three times in the last two decades to 21.13 million (6.95%). The growth from 1996 – 2010 is more than two times of the growth in the 38 years before. The burst of diabetes has boosted abundant attention and researches in these years.

Figure 1. Number and Percentage of U.S. Population with Diagnosed Diabetes, 1958–2010[10]
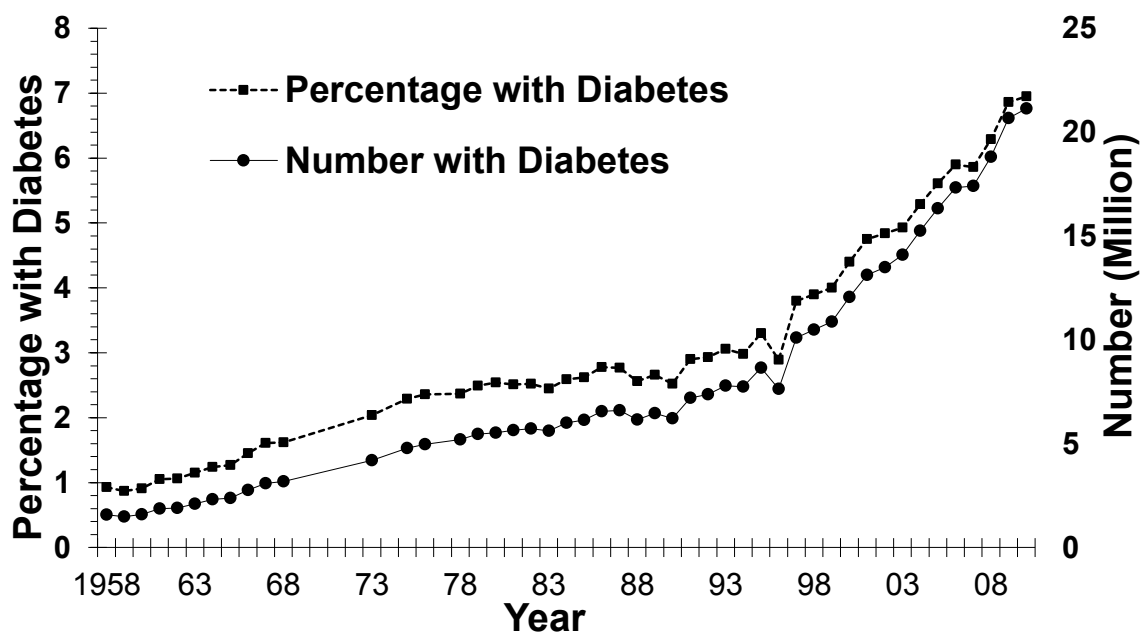
Diabetes mellitus, a series of metabolic syndromes, specialized by abnormally high level of fasting blood glucose (FBG >125 mg/dL), has become one of the most common chronic disease in the US. Diabetes is classified by causes into three main types – Type 1, Type 2 and gestational diabetes. Type 1 and Type 2 diabetes result from defective insulin production and insulin resistance, respectively. Gestational diabetes develops during pregnancy on women not previously diagnosed with diabetes.

Type 2 diabetes is the most common type, which account for 90-95% of all the diabetic cases diagnosed in the US [1]. Type 2 diabetes features insulin resistance, in which body tissues fail in response to insulin, and thus cannot efficiently utilize glucose in blood. The exact cause of Type 2 diabetes is still unknown. Some lifestyle factors can increase the risk of Type 2 diabetes, including obesity, unbalanced diet, and sedentariness. As is illustrated in Figure 2, the prevalence of diagnosed diabetes had a very high correlation with obesity. In 1994, most of states had obesity rates less than 18% and diabetes rate less than 6.0%. However, in 2010, the obesity and diabetes percentages in the majority of state were beyond 22% and 6.0% [7], respectively. Figure 3 illustrated most of the main factors correlated with diabetes. In 2010, among the diagnosed diabetic people, 56.9% were obese, 84.7% were overweight or obese, 36.1% considered themselves inactive, 57.1% reported hypertension and 58.4 reported high cholesterol level.

Diabetes can cause damage on many organs and cause a series of complications including hypoglycemia and hyperglycemic crisis, microvascular disease, high blood pressure, high blood LDL cholesterol, heart disease, eye problems, kidney disease and low-limb amputations[1]. In CDC's report, 71% of diabetic people had higher blood pressure than the normal range, 65% of diabetic

people had abnormally higher LDL cholesterol, and 60% of lower-limb amputations among adults were associated with diabetes[1]. Diabetes was also listed top of causes of kidney failure, which responsible for 40% of the cases [1] High blood pressure and

Figure 2. Prevalence of Obesity and Diagnosed Diabetes among U.S. Adults [10]

Obesity (BMI ≥30 kg/m²)
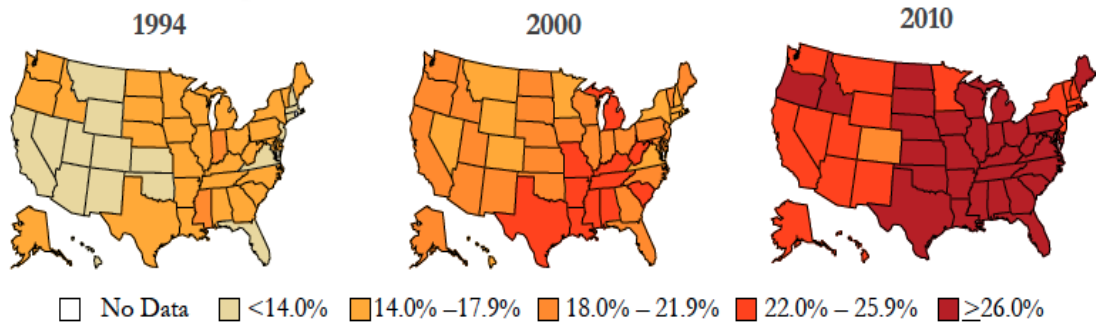
Diabetes
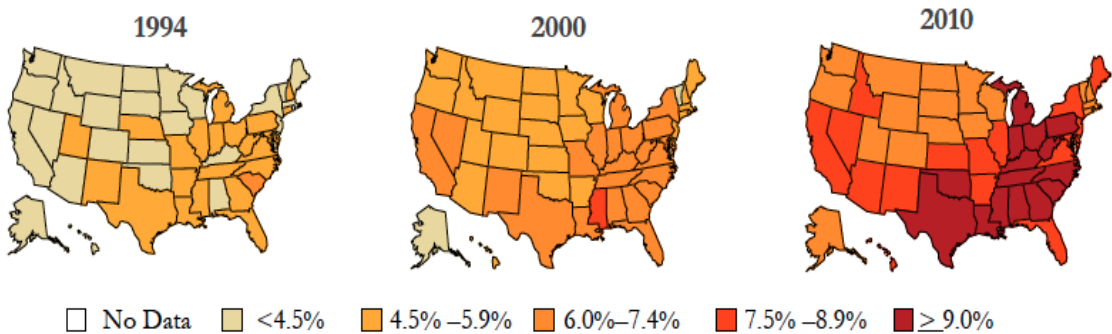
Figure 3. Age-Adjusted Percentage of Adults Aged 18 Years or Older with Diagnosed Diabetes Who Have Risk Factors for Complications, United States, 2010[11]
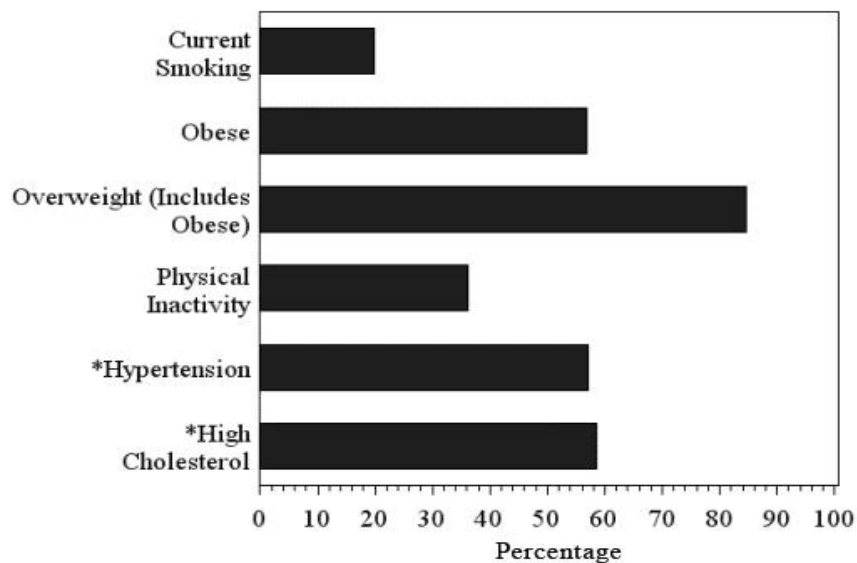
* indicate data in 2009.

LDL cholesterol levels associated with diabetes had tremendously increased the risk of heart disease or stroke. In 2010, the heart attack and stoke rates were 1.8 times and 1.5 times higher than the population without diagnosed diabetes [1]. Diabetes was also listed seventh as causes of death [1].Diabetes also leads to severe finical burden to individuals and families. The estimated cost of diabetes in 2012 was $245 billion including $176 billion direct cost on medical care of diabetes or its complications and $69 billion indirect cost on job loss, disability or premature death [1]. The average expenditures of diabetic patients were around $13,700 per year, which was 2.3 times higher than those without diabetes [1].

Type 2 diabetes usually starts from prediabetes, a status with a higher fasting blood glucose level (100-125 mg/dL) than normal but not reach the FBG level for diabetes. Usually referred as impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), prediabetes can greatly boost the risk of type 2 diabetes. In a report by CDC [2], 15% - 30% of prediabetic people may develop type 2 diabetes in three to five years. In 2009 - 2012, almost 37% of people over 20 years old have prediabetes accompanied by 12.3% already diagnosed with diabetes [1,2]. In the age group above 65 years old, people with prediabetes account for 51% [2] (Figure 4).

Figure 4: Prediabetic and Diabetic Percentage in U.S. citizens [1,2]

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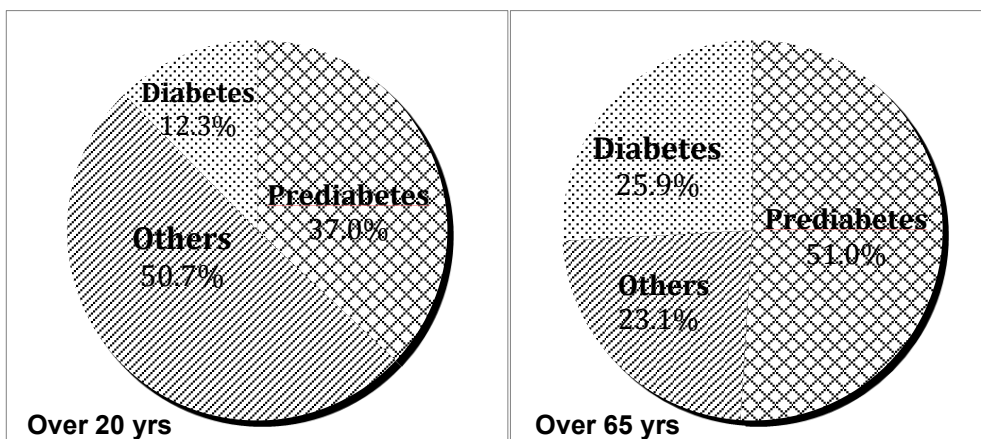
Early identification of prediabetes can proactively prevent development of type 2 diabetes through diet control, activity, and a series of life style change. The majority of people with prediabetes were not aware of their health status [2,12].

The risk of prediabetes is not limited on diabetes. It is widely reported that prediabetes is a risk factor for cardiovascular disease and neuropathy [12,13], Kurihara *et al.* [14] reported more advanced coronary atherosclerosis in prediabetic people than in normal people and comparable between prediabetic and diabetic people. Both the two indexes of atherosclerosis, total yellow plaques per vessel and maximum yellow grade, were significantly higher in prediabetic group than in nondiabetic group (P=0.02 and P=0.04, respectively), while comparable between prediabetic group and diabetic group (P=0.44 and P=0.21, respectively). There is also a positive association between insulin resistance and arterial stiffness in prediabetic people [15]. The impact of prediabetes on neuropathy is still debatable for now. Papanas *et al.* [13] reported that the peripheral neuropathy or neuropathic pain of prediabetic people as an intermediate state between apparent diabetic neuropathy and normal state. The prediabetic neuropathy mainly affects small fibers regulating sensory functions and is usually milder than diabetic neuropathy.

Functional Starch

Lifestyle intervention, including diet control and physical activities, in prediabetic people has been proved to prevent or delay type 2 diabetes.

Starch represents the major source of available carbohydrate in the human diet. There are two structures of starch defined by its linkage. Amylose is the starch molecule specified by highly linear structure linked by alpha 1→4 linkage, while amylopectin have more alpha 1→6 linkages, which make it highly branched and

easily hydrolyzed. The ratios of amylose and amylopectin vary largely in starches from different sources, which generate diverse starch structure, physiological effects and digestibility.

For nutritional purposes, starch is also classified into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) [16]. RDS is the starch fraction that is converted to glucose by enzymes in 20 min. RDS is mainly amorphous starch in food cooked in high moist conditions, such as baked potato and bread. The category of SDS includes enzyme inaccessible starch and raw starch that is fully hydrolyzed *in vitro* during prolonged incubation (20–120 min). SDS may include both amorphous and crystalline starch that cannot be digested immediately in small intestine for its granule sizes or retrogradation. Resistant starch is the starch fraction that is not hydrolyzed after 120 min incubation with α–amylase and pullulanase and calculated by subtracting SDS and RDS from the total starch content [3,17]. SDS and RS fractions are usually considered as functional starch. The low digestibility of SDS and RS can attributes to granule size, amylose ratio, processing conditions. Resistant starch family is further categorized into 5 different types by their resistant mechanisms [3,18].

Resistant starch 1 (RS1) protects itself from enzyme hydrolysis by granule size and physical structures. Most of the RS1 are coated with cellulose (cell wall) and proteins. Since cellulose and some kinds of proteins are not enzymatically hydrolyzed, amylase cannot reach the inside starch part.

Resistant starch 2 (RS2) is natural form of resistant starch found in some plants due to its natural granule form. Starch granule can swell during cooking process and is easily unfolded by water, which consequently provides more space for amylase to act on. However, in these raw starch granules, starch can be packed

very tightly which prevent hydrolysis during digestion and greatly reduce the number of sites enzyme can act on. Bananas, high-amylose corns and uncooked potatoes are good source of RS2.

Resistant starch 3 (RS3) is known as retrograded starch formed by low temperature and long-term storage. As is introduced, amylopectin parts of starch are unfolded by water and starch become more soluble when heating in solution, which is defined as gelatinization. When gelatinized starch is stored in a low temperature, the starch molecules tend to become a special crystalline form insoluble retrograded starch. The retrogradation is not reversible, which means the RS3 crystal is not gelatinized through reheat. Cold legumes, long-term-preserved bread, and sushi rice are all great source of RS3.

Resistant starch 4 (RS4) is a series of chemically modified starch, in which any bonds other than 1→4 linkage or 1→6 linkage can form, resistant to digestibility for specific purpose. RS4 has been utilized in bread and cake for health or taste purpose.

Resistant starch 5 (RS5) is an amylose – lipid complex. The attachment of lipid greatly reduces starch's capability of swelling and makes it hard to be digested.

Health Benefits of Functional Starch

Resistant starch has proven to be a great contributor in colonic fermentation. RS can stimulate the fermentation in the large intestine to produce short chain fatty acids [19,20]. In the situation that most of Americans do not intake adequate dietary fiber, resistant starch hereby provide the essential energy source for fermentation in the colon. As estimated, less than 3% of the US adults consume the recommended dietary fiber intake [19]. The average adult dietary fiber intake is only 15 g/day, while

the recommended intake is 25 g/day for women and 38 g/day for men [19]. Murphy *et al.* [22] reported that the average resistant starch intake is approximately 4.9 g/day (ranging from 2.8 to 7.9 g). As for colonic fermentation, resistant starch has worked to make up for the dietary fiber shortage. Short chain fatty acids including butyrate, acetate and propionate, have been known to be beneficial to gastrointestinal health and reduce the risk of colon cancers [23]. Animal studies revealed that RS may control the initiation of colon cancer with the stimulation of short chain fatty acids, particularly butyrate [24]. Research showed that an intake of resistant starch by healthy humans resulted in modulation of gut microbiota composition [25]. *Bifidobacterium adolescentis* and *Parabacteroides distasonis* were significantly increased with RS4 intake [25].

RS was also widely reported for its health benefits in blood glucose control and diabetes' prevention. Since RS is not digestible within small intestine, food rich in RS can prevent a sudden rise of postprandial blood glucose and insulin. Human study demonstrated that the RS intake could significantly reduce postprandial plasma glucose, insulin and satiety hormones including gastric inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [26]. Kendall *et al.* [27] specifically reported that a maize-base diet rich in resistant starch greatly decrease the postprandial blood glucose and insulin level within 120 min. The blood glucose lowering effect of RS may also contribute to the increase of satiety led by RS intake. A study also elucidated that RS rich meal might increase the satiety compared with a non-RS control diet [28]. A supplementation of 25 g/day of RS3 in healthy subjects caused a laxative effect with no significant gastrointestinal discomfort [29]. Some manually modified resistant starch (RS5) has been reported its effect in managing blood glucose and insulin responses. Hasjim *et al.* [30] showed that a novel resistant starch,

which synthesized through complexion of high-amylose maize starch and palmitic acid, significantly reduced postprandial blood glucose and insulin levels to 55% and 44% compared with control, respectively.

The studies on the nutritional qualities of SDS are limited. Moreover, most studies could not make a clear distinction on the health benefits of different starch fractions in a specific food item. The potential health benefits of SDS resemble that of RS in glycemic response, satiety controls and diabetes prevention.

SDS were reported to have a medium to low glycemic index (GI) while RDS usually have a very high GI because of its rapid release of glucose [31]. Foods rich in SDS were reported for a significantly lower glycemic load when compared with foods high in RDS with a higher GI [31, 32]. Significant reduction was observed in blood glucose, insulin after the consumption of SDS than consumption of RDS in a few studies [31, 33]. According to Mayer's theory [34], low blood glucose could trigger feeding manner and high blood glucose could trigger satiety. Based on this theory, the consumption of food rich in SDS can maintain a prolonged glucose and insulin response, which may increase satiety. Though studies on the direct relation of SDS and satiety are limited, the significant findings of SDS on glycemic control make it an important study focus on diabetes prevention.

Grain Sorghum

Grain sorghum is the main staple foods that feed a great population in vast arid and semi-arid area. Though grain sorghum is not very widely consumed in the US, it is the third most abundant crop grown in US and the fifth most important crop grown in the world in production [5]. Grain sorghum is commonly consumed in Africa, and Asia in many food products. The drought tolerant properties of grain sorghum

make it the only possible staple in some areas, whereas in some countries including United States, grain sorghum is grown for animal feed due to its high production [35]. Though the production of sorghum is growing these years, the majority of sorghum production is for animal feeding, and applications like ethanol production. In the US, due to sorghum's poor protein digestibility, around 90% of sorghum is used as livestock feeding [36, 37].

Sorghums have a common structure similar to wheat. The grain of sorghum contains a pericarp, the testa between pericarp and endosperm, the endosperm, and the embryo (Figure 5). Normal (nonwaxy) grain sorghum has a similar total starch content as wheat, ranging from 60 – 75% total starch [5,8] and 14 – 31% of the starch is amylose [38]. Special grain sorghums including waxy sorghum and high amylose sorghum, however, were fostered for extremely high amylopectin and amylose content, respectively. The amylose content in waxy sorghum can be as low as 0 - 5% [39, 40], while high amylose sorghum may contain up to 70% amylose [38]. The average gelatinization temperature of isolated starch from sorghum ranges from 75.3 to 78.4°C [41].

Figure 5: Structure of sorghum grain [42]
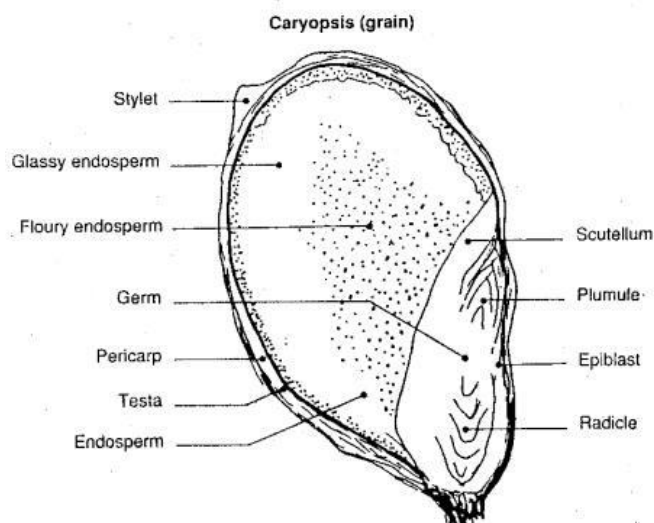
Grain sorghum usually has the lowest starch digestibility among all cereals. In some research, a similar starch digestibility was reported between sorghum and corn isolated starch for their biological equivalence [39]. Factors affecting sorghum starch digestibility may include starch-protein matrix, amylose content, presence of tannins, endosperm texture (vitreous or floury), and cooking conditions.

Most of the starch exists in granule form embedded within a protein matrix. Although grain sorghum has similar chemical composition compared with wheat, sorghum contains higher content of resistant starch (RS), which affects its digestibility [4]. Raw grain sorghum contains 40 – 65% of RS, while the RS content of cooked sorghum ranges from 5-20%, correlated to their cultivars [43].

Some sorghum cultivars contain high level of tannin, which may decrease the activity of α-amylase and thus reduce the digestibility of starch in whole sorghum products. Tannin is a polyphenolic compound unique in sorghum among all cereals. Condensed tannin isolated from sorghum grain has showed to inhibit α-amylase activity and hence, reduce starch digestibility [44]. Tannin can also interact with amylose and linear parts of amylopectin in starch and decrease starch digestibility [45]. The addition of isolated sorghum tannins to wheat, corn, and sorghum starches in different concentration showed the tannins' inhibition on α-amylase [44]. Foods made from high tannin sorghums varieties have comparatively lower starch digestibility [43, 44].

Starch-protein interaction also exerts a strong influence in sorghum's starch digestibility. Grain sorghum has a protein range of 7 to 15%. Among the varieties of proteins, a prolamin (alcohol soluble protein) - kafirins comprise about 50-70% of the proteins [47-49]. The kafirins, mainly in endosperm, wrap the starch granules and greatly inhibit water penetration and enzymatic digestion. The presence of kafirins

significantly decreases the digestibility of sorghum starch.[50,51] . The kafirins can be further categorized into three groups, α-kafirins (22-25kDa), β-kafirins (16-20 kDa) and γ-kafirins (28-29 kDa). The γ-kafirins were usually distributed at periphery of protein bodies. γ-kafirins can become more rigid after heating process due to stronger intramolecular disulfide cross bonding. The addition of 2-mercaptoethanol, a reducing agent, could prevent the formation of disulfide bond in heating and significantly enhance starch digestibility, which also proved the influence of kafirin during cooking [52]. In 1981, Axtell *et al.* [50] found that the cooking process greatly reduce sorghum's protein digestibility from 78-100% to 45-55%. Other studies also showed a significantly lower starch digestibility in cooked sorghum grain [51, 53].

The reduction in starch digestibility was mainly contributed to cell wall material, endosperm protein matrix, and tannins' presence. Other than kafirins, protein barrier around starch granule could also hinder amylolytic enzymes and reduce starch hydrolysis [39]. The addition of pronase, however, could hydrolyze the protein matrix and significantly increase *in vitro* starch digestion by increasing surface area of starch interacting with α-amylase and amyloglucosidase [39]. The presence of unique starch-protein matrix has been proved as the primary cause of low feeding quality of sorghum compared with corn [39].

High amylose content is usually related to low starch digestibility. Some low amylose sorghum cultivars (waxy sorghum) showed a much higher *in vitro* digestibility by glucoamylase than normal sorghum[54]. Wong *et al* [55] also reported that besides high kafirin content, a higher amylose content of sorghum starch, might also have a great impact on the digestibility of both starch and protein in sorghum grain endosperm.

Other factors including endosperm texture (vitreous or floury) and cooking conditions can also affect sorghum's *in vitro* starch digestibility of grain sorghum [56]. Vitreous endosperm could suppress starch digestibility by blocking the access of α-amylase through its prolamin protein network [56]. The tough peripheral endosperm layer of whole grain could also reduce starch digestibility by wrapping starch granule and prevent enzyme access [39, 57].

Health Benefits of Grain Sorghum

Grain sorghum is widely used as animal feed in America. The utilization of grain sorghum for human consumption is still uncommon. Due to its high RS concentration and the presence of starch-protein matrix, grain sorghum shows a potential as a staple for those population, which requires energy control, diabetic prevention and gluten free diet. The human research on grain sorghum is still rare. Poquette *et al.* [64] reported significant postprandial blood glucose and insulin reduction with the consumption of grain sorghum muffins compared with control wheat muffins in 10 healthy men. The glucose level was significantly lower in sorghum treatments at 45 – 120 min intervals while insulin response was significantly lower at 15 – 90 min intervals. Incremental area under the curve (iAUC) was 35% lower for glucose response and 55% lower for insulin response with sorghum treatments. Further human studies on grain sorghum from different cultivars on different health conditions are needed.

Materials and Methods

Materials

Whole grain sorghum flour from Bob's Red Mill (Milwaukie, OR, U.S.A.) and whole wheat flour from Gold Medal (Minneapolis, MN, U.S.A.) were purchased from local grocery stores. Solvents for chemical analyses in the experiment were purchased from VWR international, Inc. (Suwanee, GA, U.S.A) and Sigma Chemical Co. (St. Louis, Mo., U.S.A). A total starch kit was purchased from Megazyme International Ireland Ltd. (Bray Business Park, Wicklow, Ireland). An insulin ELISA kit for plasma insulin determination was purchased from Mercodia (Uppsala, Sweden).

Participant Profile and Study Design

All participants were recruited from University of Arkansas (Fayetteville, AR, USA) and surrounding area. Study procedures were approved by IRB at University of Arkansas. Fifteen male subjects, 18-45 year of age, participated in a screening session to sign a consent form and a screening form. Their fasting blood glucose levels (100-125 mg/dL) were confirmed with an Accu-Chek Aviva Plus glucometer (Roche, Indianapolis, IN). During a screening session, we also recorded subjects' height and weight for Body Mass Index (BMI) calculation and confirmed that participants were not diagnosed with any disease and not taking any medication, which might affect blood glucose levels. Participants must be non-smokers, and did not consume more than two alcohol servings per week. Participants were assigned into different study cohorts consisting 3 – 4 subjects in different weeks. All subjects in each cohort were randomly assigned for either grain sorghum or wheat treatment on the first week and were served with the other treatment on the following week with a one-week washout period. Participants arrived at 7:45 am with at least 10-

hour fasting. Muffins contained 50 g of total starch from either grain-sorghum or whole-wheat flour, which the subject consumed at breakfast.

Muffin Preparation

Muffins containing a total serving of 50 g of total starch were prepared from whole wheat or grain sorghum flour. Raw materials for muffins were weighed in different bowls as shown in Table 1. Dry ingredients and wet ingredients were mixed separately and then combined. Aliquots from grain sorghum and wheat doughs containing 25 g of total starch were weighed into greased muffin liners. Both sorghum and wheat muffins were baked at 425 °F for 15 minutes in the same tray. Muffins were cooled for 15 min and stored at room temperature in a plastic muffin container with cover for the experiments in the following mornings. One muffin from each treatment was minced with a Farberware food blender (Meyer, Vallejo, CA) for exactly 30 s with a pause at 15 s and then was analyzed on total starch (TS) content and starch fractions (RDS, SDS, and RS).

Each subject was instructed to eat two muffins and 250 mL of water for each treatment within 7 min on respective experiment days. All ingredients were purchased at a local grocery store.

Moisture Content Determination

Moisture content of flours and muffins were determined following Moisture – Air – Oven (Aluminum-Plate) Method [55]. Approximately two grams of minced muffin and flour samples were placed in 55 mm diameter aluminum pans. Pans were weighed and recorded empty and with samples. The samples were dried in a convection oven (VWR, Suwanee, GA) at 130 °C for 2 hr. After the drying period,

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the samples were transferred to a desiccator to cool to room temperature for 30 minutes and were weighed to determine the weight loss. The moisture percent was interpreted as noted in Equation (1):

$$\% \ Moisture \ = \frac{loss \ of \ moisture \ \times \ 100}{wt \ of \ sample} \qquad (1)$$

Total Starch Content Determination

Total starch of raw flour samples and muffin was processed and determined following the manual (KOH format) (Megazyme, Bray Business Park, Wicklow, Ireland). Briefly, flour and muffin samples (100 mg) were dissolved in 2 M KOH in an ice-water bath to solubilize RS. The flour solutions were then added with sodium acetate buffer (1.2 M, pH 3.8) followed by immediate addition of 100 μL of thermostable α-amylase and 100 μL of amyloglucosidase. After 30 min incubation at 50 ℃, each sample was transferred and diluted in a 100 mL volumetric flask. Small aliquots (0.1 mL) were added into 3 mL of glucose oxidase plus peroxidase and 4-aminoantipyrine (GOPOD) reagent and incubated at 50 ℃ for 20 min. A blank and a standard (1mg/mL glucose solution) were carried out with samples. The absorbance was read at 510 nm against blank to determine the TS content. The starch content was calculated as noted in Equation (2):

$$S = \Delta A \ \times \ \frac{F}{W} \ \times \ 0.9 \ \times \ FV \ \frac{100}{100 - moisture \ content \ (\% \frac{w}{w})} \qquad (2)$$

Where

S = the starch content percentage on a dry basis

ΔA = absorbance read against the reagent blank

F = the conversion from absorbance to μg

FV = final volume (100 mL)

W = the weight in milligrams of the flour/muffin

Starch Fractions Determination

RDS, SDS, and RS were determined at the same time when subjects consumed muffins. Enzyme solution preparation: 450 mg of pancreatin from porcine pancreas (Sigma, St. Louis, MO) was soaked in 20 mL of deionized water with stirring for 10 minutes and after centrifugation at 1500 x g, 54 mL of supernatant was mixed with 6 mL of amyloglucosidase (140 unit/mL) from Aspergillus niger (Sigma, St. Louis, MO). Muffin samples were broken in small pieces by hands and then minced with a Farberware food blender (Meyer, Vallejo, CA) for exactly 30 s with a pause at 15 s. Minced muffins were weighed in centrifuge tubes on the basis of starch content, which were calculated to be the same as 800 mg of flour for both grain sorghum and wheat. 20 mL of sodium acetate buffer (0.5 M, pH 5.2) and 5 mL of prepared enzyme were added to the tubes and mixed well. All the tubes were incubated horizontally in water bath at 37 ^{0} C. At 20 min, an aliquot of 0.5 mL was pipetted from tube to 20 mL of 80% ethanol and mixed well for glucose determination (G20). Samples were replaced into water bath within 1 min for time accuracy. At 120 min, another aliquot of 0.5 mL was pipetted from the tube for glucose determination (G120). A standard (20 mL of 25 mg/mL D-glucose in sodium acetate buffer) and a blank (20 mL of sodium acetate buffer) were treated in the same condition as samples. A supernatant of 0.1 mL was mixed with 3 mL of GOPOD reagent followed by incubation at 50 ℃ for 20 minutes. The absorbance at 510 nm was read and RDS

and SDS were calculated according to Englyst *et al*.[3]. The RS was calculated by subtracting RDS and SDS from TS.

Amylose Content Determination

Amylose content of flours was determined following AACC method (Amylose content of milled rice) [59]. Duplicate samples of 100 mg were dissolved in 1 mL of 95% ethanol and 9 mL of 1 N NaOH overnight. The samples were then diluted in a 100 mL volumetric flask. An aliquot of 0.5 mL was taken and mixed with 5.0 mL water and 0.1 mL 1 N acetic acid. The solution was then added with 0.25 mL of IKI solution (0.2% I_2 in 2% KI) and dilute to 10 mL with distilled water. The samples was incubated for 30 min before the absorbance at 620 nm was read against blank. Standards containing 0%, 5%, 15%, 25%, 35% amylose were prepared by mixing high amylose potato starch and waxy rice starch. A standard curve was made to calculate the amylose content in flours.

Protein Determination

The crude protein of muffins and flours were determined with the Micro Kjeldahl Method [57] using a Micro digesters and kjeldahl distillation system (Labconco, Kansas City, MO). Flours and Minced muffins were completely digested in 5 mL concentrated sulfuric acid with ½ pill of Kjeldahl catalyst tablet for Wieninger (EMD Millipore, Jaffrey, NH) until the solution is clear green. The digested samples were then distilled and titrated with HCl. The starch content was calculated as noted in Equation (3):

$$\%Protein = \frac{(S - B) \times N \times 1.4007 \times f}{sample wt.(d.b.)} \qquad (4)$$

Where

S: mL alkaline back-titration of sample

B: mL alkaline back-titration of blank

N: normality of acid

f: convention factor, f=6.25 for grains

Lipid Determination

The crude fat in muffins was determined with a Soxtec Avanti 2055 system (Foss North America, MN, US) [61.] The ground muffin samples were weighed into porous thimbles and extracted in ether, and lipid was collected in extraction cups. After the evaporation of solvent and drying process, lipid was weighed and the crude fat content was calculated.

Kafirin and Gliadin (Prolamin) Determination

Protein fractions for both sorghum and wheat flours were determined following the method introduced by Wallace et al [62]. Briefly, 200 mg of each flour samples were divided into three portions in several steps. Nonprotein nitrogen, albumin and globulin proteins were washed with 0.5 M NaCl solution at 4 ℃ in advance. The flours were then extracted with 2 mL 1% sodium dodecyl sulfate, 12.5 mM sodium borate, 2% 2-mercaptoethanol (2-Me) (pH=10) on a shaker for 1 hour. The mixture was then centrifuged at 5000 x g for 20 min. The extraction were repeated twice and the supernatants were combined and t-butanol was added to form a final concentration of 60%. The samples were allowed to precipitate for 2 hours and centrifuged at 5000 x g for 20 min. The supernatant was prolamin and the precipitation was nonprolamin (glutelin). The three fractions: non-protein nitrogen

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(NPN), prolamin and nonprolamin (glutelin) were dried at 80℃ and analyzed with the

Micro Kjeldahl Method [57] introduced above.

Plasma Glucose and Insulin Analysis

Human blood samples were collected at 15 minutes before (baseline) treatments and 0, 15, 30, 45, 60, 75, 90, 120, and 180 minutes after the consumption of muffins. Each subject was reminded 2 min before each blood draw time point to warm up hands for the ease of bleeding. Around 0.4 mL of blood was drawn with 6 capillary tubes and centrifuged at 4000 x g with Microfuge® 22R Centrifuge (Beckman Coulter, Inc., Brea, CA, U.S.) to collect plasma. The exact time points of starting and finishing blood draw were recorded to ensure every blood draw was within 4 min. Plasma glucose levels were determined with an ACE AleraTM Clinical Analyzer (Alfa Wassermann Diagnostic Technologies, LLC, West Caldwell, NJ). An ELISA kit from Mercodia (Uppsala, Sweden) was used for plasma insulin determination. The incremental area under the curve (iAUC) was calculated by the Trapezoidal rule [63]. The iAUC was calculated as noted in Equation (4):

$$iAUC = \sum_{i=1}^{9}(X_i + X_{i+1}) \times (T_{i+1} - T_i)/2 \qquad (4)$$

where

X_i= Glucose or insulin readings at different time points

T_i= Time before or after the consumption of muffins

i= Time points (-15 min – 180min)

Statistical Analysis

All statistical analyses were conducted with the Statistical Analysis System (SAS, Release 9.4, SAS Institute Inc, Cary, NC). Values were expressed as means ± standard error of the mean (SEM). The significance of starch fractions and protein fractions between grain sorghum and wheat were analyzed by two sample t-test. The significance of differences among incremental mean values of glucose and insulin on grain sorghum and wheat treatments were compared with paired t-test. The incremental area under the curve (iAUC) of glucose and insulin between two treatments were analyzed with paired t-test. The time trend correlations of each treatment on both incremental blood glucose and insulin responses were analyzed with PROC GLM (repeated statement), with the comparison of incremental glucose and insulin response on each time point. Differences were considered significant at $P < 0.05$.

Results and Discussion

Flour and Muffin Starch Analysis

Grain sorghum flour contained 28.0 ± 0.3, 45.9 ± 0.0, and 26.1 ± 0.0 % for RDS, SDS and RS, respectively. The control, wheat flour, contained 21.3 ± 0.1, 20.9 ± 0.0, and 57.9 ± 0.0 % for RDS, SDS and RS (Table 2). Concerning functional starch, raw grain sorghum flour showed significantly higher SDS content and lower RS content than wheat flour in this study. The RS content of control flour was around two times of that of sorghum flour. The majority of sorghum flour was SDS, which was 119.6% higher than wheat flour. Grain sorghum flours were mostly specialized for their lower digestibility than other cereals like wheat, rice, maize, barley and oat [64, 65]. The total starch contents were 77.5 ± 0.7% for sorghum flour and 72.5 ± 1.0% for wheat flour. Grain sorghum TS content was similar to other studies [7, 66]. Austin *et al.* [66] reported 74% TS of Texas white sorghum. Various factors could affect starch digestibility in grain sorghum, including starch protein interaction, tannin contents, amylose content, endosperm structure, and processing and cooking conditions. In the present study, the amylose contents of grain sorghum flour and wheat flour were 27.9 ± 1.4% and 20.5 ± 0.1%, respectively. The amylose content in the present is close to other studies [5, 8, 63]. Dicko *et al.* [5] reported 65 – 70% total starch content and 12 – 22% amylose content in grain sorghum. Sang *et al.* [8] found the grain sorghum containing 24% amylose content. In two Nigerian sorghum cultivars, Gaffa *et al.* [67] reported 25.5% and 25.7% amylose contents. The slightly higher amylose content in grain sorghum flour was not correlated with the lower RS content in this study.

After a series of cooking process, sorghum muffins measured a TS content of 58.4% while wheat muffins measured 52.9%. The differences of TS% in two different muffins were caused by different final weight. Due to a higher TS in raw sorghum

flour, approximately 5 g more wheat flour was added to the control treatment to ensure the same TS content (50 g of starch) in both sorghum and wheat muffins. Moreover, more water was added to provide acceptable texture for wheat muffins, which also increased the muffin weigh and led to a slightly lower TS% in wheat muffins. Notably, although both sorghum and wheat flours were purchased from the same companies as described in a study conducted by Poquette *et al.* [64], the starch fractions of wheat flour in the present study showed higher SDS and RS contents compared to their report [64] (RDS: 37.5 ± 0.2; SDS: 47.4 ± 0.3; $15.1 \pm 0.1\%$). The different starch fractions of wheat flours between these two studies might result from different growing conditions (climate, seasons, and soil), processing and storage conditions, or other environmental factors.

Compared with wheat muffins, the starch fractions of sorghum muffins showed lower RDS% and higher functional starch (SDS+RS) contents. Grain sorghum muffin contained 68.2 ± 1.1, 10.4 ± 1.2, and 21.4 ± 1.5 % for RDS, SDS and RS, respectively. The control muffin contained 76.8 ± 1.3, 5.9 ± 1.9, and 17.3 ± 1.6 % for RDS, SDS and RS (Table 3). As is shown in Table 3, the functional starch content of grain sorghum muffin was 137.1% of control muffin.

The higher functional starch content in grain sorghum was very likely caused by intense starch protein matrix in sorghum fortified by wet-heating process. Starch granules in grain sorghum are usually connected or wrapped in protin bodies. The kafirin protein is the main protein fraction in protein bodies that interact with starch granules in corneous endosperm [68, 69]. β- and γ-kafirins made the most contribution to starch indigestiblity and during the cooking process, disulfide-linkage between small α-kafirin polymers was stimulated and formed more β- and γ-kafirins [49, 70]. As indicated in Table 4, the majority of sorghum protein is prolamin (kafirin), accounting

for 76.41 ± 0.25% of total protein, while the prolamin (gliadin) was only 45.59 ± 1.22% in wheat protein. A part of starch in grain sorghum was wrapped in protein body, which restricted the access of emzyme in digestion in sorghum muffins. In contrast, the majority of functional starch in raw wheat flour was lost during wet-heating process. The difference in digestibility between grain sorghum and wheat was widened during cooking, which can make it a good candidate for energy control for patients with metabolic syndromes.

Subject Characteristics

In the present study, 15 prediabetic male subjects were recruited and completed the study. Table 5 shows the subject profile of the study group. Fifteen subjects identified themselves as either: African/African American, Asian/Asian American, or Caucasian, and subjects represented either the normal or overweight BMI (Body Mass Index) category.

Dietary Assessment

Food frequency questionnaires (FFQ) were collected to measure the nutrient intake of all subjects, and then analyzed by Nutritionist Pro (Axxya Systems, WA). All subjects had a typical American diet ratio (Table 6). Around half of energy were from carbohydrate; 35.4% energy were from fat, while protein was 16.3%. According to the questionnaire, most of our subjects' diets were not very balanced. The average daily intake of dietary fiber was inadequate (22.7 ± 2.1 g) for almost all subjects, ranging from 10 to 38 g/day. The recommended fiber daily intake for adult men is 38 g/day (DRIs). The DRIs recommended a dietary cholesterol intake of less than 300 mg per day, while our subjects consumed 386.1 ± 77.1 mg/day. The average

saturated fat intake was 33.2 ± 5.0 g/day (298.8 ± 45.0 Kcals), which is also slightly above recommended value: 240 Kcals (10% of total energy intake). The average monounsaturated fat and polyunsaturated fat intakes were 34.9 ± 4.7 g/day and 23.4 ± 4.0 g/day, respectively. More polyunsaturated fat intake would be recommended. Those factors, including high fat, high cholesterol and low dietary fiber intake, might not be helpful for the prevention of diabetes.

Incremental Plasma Glucose and Insulin Responses after Consuming Muffins

As is illustrated in Figure 6, participants showed significantly lower glucose responses at 45, 60, 75, 90 and 120 minutes with the sorghum muffin treatment (P<0.05). The grain sorghum treatment induced a more gentle overall glucose response during the 180 minutes study, which implied a lighter glucose burden to those prediabetic subjects. Prediabetic subjects also showed a delayed glucose scavenging when compared with healthy subjects[64]. The grain sorghum muffins seem to be more effective in postprandial blood glucose control on prediabetic men than in healthy men [64]. In healthy men [64], the numeric maximum response of blood glucose for both grain sorghum and wheat treatments appeared at 30 min and immediately started to drop back to baseline. In prediabetic men, the postprandial blood glucose response of sorghum treatment rose to the peak until 30 min and dropped down back to baseline slowly from 30 – 180 min, while the wheat treatment showed a prolonged high blood glucose level from 30 – 120 min (P<0.05). The gaps between the average glucose incremental values of grain sorghum and wheat treatments in healthy men were smaller than that of prediabetic subjects at 45, 60, 75, 90 and 120 minutes. The difference of postprandial blood glucose responses between these two health groups (heathy *vs* prediabetic) with similar muffin

treatments might be caused by difference insulin sensitivities. Chou *et al* [15] reported that early insulin sensitivity decayed in prediabetic people. Compared to the control (wheat muffin), the insulin responses for the sorghum muffin treatment in prediabetic men were significantly lower at same time points: 45, 60, 75, 90 and 120 minutes ($P<0.05$) (Figure 7). The ranges of incremental blood insulin level for sorghum and wheat muffins were 25-40 mU/L and 45-60 mU/L, respectively. The insulin responses of grain sorghum and wheat treatments showed very different patterns in healthy men [64]. The average incremental insulin response on grain sorghum treatment remained low (0 – 20 mU/L) because of less glucose digested from sorghum muffins (lower RDS content) [64]. The wheat treatment induced a much larger amount of blood insulin (0 – 40 mU/L) from 0 – 30 min, which might be correspondent to higher RDS content in wheat muffins [64]. Despite the high level of RDS in wheat muffins, blood glucose levels were similar in both treatments. This might be due to the high insulin sensitivity in health men. In the present study, prediabetic men required larger amount of insulin (low insulin sensitivity or insulin resistance) in order to keep blood glucose stable (approximately two-fold greater than healthy men). The postprandial blood glucose and insulin responses were also correlated with the *in vitro* starch digestibility. Grain sorghum and wheat muffin consumptions showed similar glucose and insulin responses at early time points (0, 15, 30 min), which could be interpreted by the *in vitro* starch digestibility test. Wheat muffins contained more RDS and might have caused the immediate postprandial blood glucose increase (0 – 45 min), which retained until 45 min before enough insulin was secreted to help cells to respond the high glucose level. Through 45 – 120 min, grain sorghum muffins showed significantly lower blood glucose level, which was also in accordance with slower digestion and lower digestibility of

sorghum. A maize-base diet with low *in-vitro* digestibility was reported to greatly decrease both postprandial blood glucose and insulin level from 15 – 120 min [27], which also agreed with our finding. A diet treatment with low GI (glycemic index) wheat bread significantly reduced the postprandial blood glucose from 30 – 95 min and the postprandial insulin area under the curve (AUC) was reduced by 35% [71]. Soong et al [72] conducted a study to compare the *in vitro* starch digestibility in several common cereal muffins and found that rice and wheat showed a significantly higher *in-vitro* digestibility compared with oat, corn and barley. Bao *et al* [73] reported that long-term consumption of oat, which is also high in functional starch and dietary fibers, might improve glycemic response and insulin sensitivity. Due to the self-adjustment of body by secretion of insulin and several satiety hormones, which are absent in in vitro starch digestibility experiments, the prediction from in vitro study is not always straight correlated with human responses. Kempen *et al* [74] compared the in vitro starch digestibility and glucose response in pigs. The blood glucose present in vivo was less than in vitro and the plateau of glucose response was also later in vivo than in vitro study, which is also similar to our findings.

As is illustrated in Figure 8, the mean incremental area under the curve (iAUC) of glucose was significantly reduced by 35.0%, from 5457.5 ± 645.4 to 3550.0 ± 428.9 mg (~3 h) dL^{-1} (p<0.05). The mean iAUC of insulin is correspondently reduced by 36.7%, from 7254.6 ± 1228.9 to 4589.3 ± 737.2 mU (~3 h) L^{-1} (p<0.05). A study conducted by Poquette *et al.* [64] of grain sorghum on healthy men reported an almost identical glucose reduction trend (35%)[64], while a greater decrease in insulin response (55%) [64] during the 3 hour periods. The iAUC of glucose and insulin in healthy men were reduced by 34.5% and 55%, respectively. The comparable glucose reduction in these two studies (health group and prediabetic group) reflected

the similar overall glucose burdens in healthy and prediabetic groups. The difference of insulin iAUC responses in these two different health groups might also indicate low insulin sensitivity in prediabetic subjects. Therefore, the grain sorghum treatment reduced the overall postprandial blood glucose and insulin burden. The reduction in blood glucose and insulin were more likely from lower starch digestibility on grain sorghum muffins than from enhancement in insulin sensitivity in this short-term study.

Diet with high resistant starch has been proven to control postprandial blood glucose and prevent or relieve type 2 diabetes in different experimental models in both short-term and long-term studies. In most short-term studies, diets rich in functional starch reduce temporary blood glucose and insulin burden [64, 75]. In most long-term studies, functional starch worked to improve or fix carbohydrate metabolism [76, 77]. Raben *et al*[75] reported that raw potato starch (mainly RS 2) significantly reduced postprandial blood glucose, insulin, gastric inhibitory glucagon-like peptide-1 (GLP – 1), polypeptide (GIP), and epinephrine in healthy men. For a long-term diet, animal study also showed functional starch could change satiety related hormones and prolong satiety. Silva *et al*.[76] observed reductions on both insulin and Glucagon-like peptide-1 (GLP-1) in RS-fed pigs, which indicated high RS diet might prolong satiety. Studies of functional starch's health effects on prediabetic people are still limited. A study of dietary fiber fortified bread on women with impaired blood glucose tolerance illustrated similar postprandial glucose and insulin responses as our study [71]. The consumption of low GI bread significantly reduced the postprandial blood glucose from 30 – 95 min and the postprandial insulin area under the curve (AUC) was reduced by 35% in prediabetic women [71]. The treatment did not change any of fasting heath parameters including glucose, insulin, cholesterol and triglyceride, which implied that low GI food including functional starch

might not affect glucose or insulin metabolism in prediabetic people in a short term treatment [71]. Kwak *et al* [78] reported that a four-week diet intervention with resistant starch added rice significantly reduced fasting insulin and insulin resistance in prediabetic people and people newly diagnosed with type 2 diabetes. This study also suggested that long-term consumption of resistant starch might improve glucose and insulin metabolism by protecting endothelial function from oxidative stress. Mitra *et al*. [77] reported that the long-term consumption of high RS rice could positively adjust health parameters including fasting blood glucose, total and LDL cholesterols, hence improve the whole carbohydrate metabolism[77]. In some animal studies, however, grain sorghum diet showed a higher starch digestibility, mostly on ruminant animals. Nikkhah *et al.* [79] reported a higher plasma glucose response after a processed sorghum treatment compared with a barley treatment in midlactating cows. In another study conducting on sheep, grain sorghum based diet led to a weaker glucose tolerance and lower insulin response than maize diet on the glucose tolerance test [80].

Conclusions

Results show the clinical effectiveness of the grain sorghum muffin on blood glucose and insulin management in persons with prediabetes. Compared with the control muffin, grain sorghum muffin showed significantly higher functional starch fractions, including both SDS and RS. Consumption of grain sorghum muffin reduced the postprandial blood glucose by 35% and insulin by 37% compared with the control treatment. The consumption of grain sorghum muffin can reduce the temporary blood glucose and insulin in prediabetic men and long-term consumption might help to improve the impaired glucose tolerance. Grain sorghum is a good functional starch ingredient to improve prediabetic people's health.

Table 1. Ingredients used for muffin preparation

Ingredient/muffin	Wheat Muffin (g)	Sorghum Muffin (g)
Flour	69.0	64.5
Water	41.2	36.0
Egg	28.8	28.8
Butter	20.0	20.0
Vegetable Oil	7.5	7.5
Sucralose	3.0	3.0
Baking Soda	1.8	1.8
Salt	1.3	1.3
Vanilla Extract	1.8	1.8

Table 2. Starch Fractions and Total Starch Contents of Wheat and Sorghum Flours

Flour	RDS%	SDS%	RS%	TS%
Wheat	21.3 ± 0.1	20.9 ± 0.0	57.9 ± 0.0*	72.5 ± 1.0
Sorghum	28.0 ± 0.3*	45.9 ± 0.0*	26.1 ± 0.0	77.5 ± 0.7*

All values are means ± SD; * indicate P<0.05, significance was compared in column.

Table 3. Starch Fraction and Total Starch Contents of Wheat and Sorghum muffins 34

Muffin	RDS%	SDS%	RS%	TS%
Wheat	76.8 ± 1.3*	5.9 ± 1.9	17.3 ± 1.6	52.9 ± 0.5
Sorghum	68.2 ± 1.1	10.4 ± 1.2*	21.4 ± 1.5*	58.4 ± 2.4*

All values are means ± SD; * indicate $P < 0.05$, significance was compared in column.

Table 4. Protein Fraction of Wheat and Sorghum flour

Flour	NPN (%)	Prolamin (%)	NP (%)	NE (%)	TP (%)
Wheat	22.54 ± 0.39*	45.59 ± 1.22	31.04 ± 0.74*	0.84 ± 0.86	12.84±0.14*
Sorghum	9.32 ± 0.24	76.41 ± 0.25*	11.52 ± 0.10	2.75 ± 0.17*	9.54 ± 0.07

All values are means ± SD; total protein is on the basis of dry weight of flour; all the protein fractions are on the basis of total protein; prolamins are present as kafirin in sorghum and gliadin in wheat; * indicate $P<0.05$, significance was compared in column. NPN: non-protein nitrogen; NP: stands for Non-prolamin protein; NE: stands for non-extractable nitrogen; TP: stands for total protein.

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Table 5. Subject Characteristics

Subject Number (n)	15
Ethnicity	Asian/Asian American (n=7) Caucasian (n=8)
Age (y)	30.5 ± 1.9
Fasting Blood Glucose (mg/dL)	108.0 ± 1.4
Body Mass Index (kg/m^2)	26.3 ± 1.1

All values are means ± SEM.

Table 6. Dietary Assessment

Average Daily Energy Intake (Kcals)	2380.4 ± 237.3
Carbohydrate (%)	47.3 ± 2.1
Dietary Fiber (g)	22.7 ± 2.1
Protein (%)	16.3 ± 0.7
Total Fat (%)	35.4 ± 1.8
Saturated Fat (g)	33.2 ± 5.0
Monounsaturated Fat (g)	34.9 ± 4.7
Polyunsaturated Fat (g)	23.4 ± 4.0
Dietary Cholesterol (mg)	386.1 ± 77.1

All values are means ± SEM; n = 15

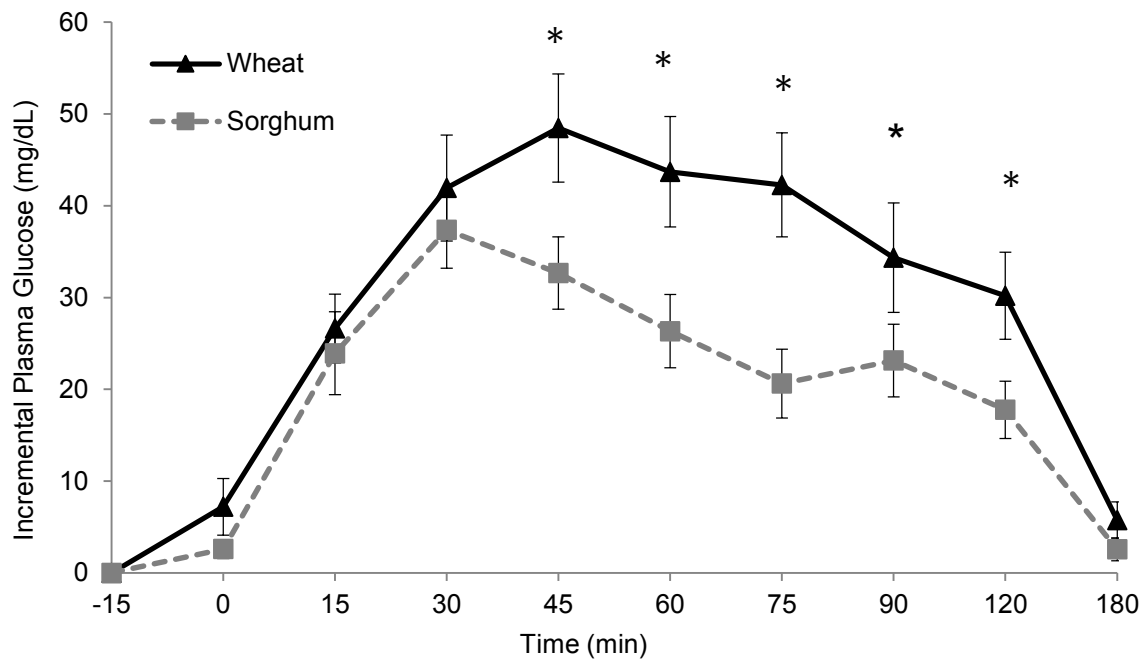Figure 6. Mean incremental change of plasma glucose concentrations in prediabetic men (n=15) with SEM; * indicate $P<0.05$, significance was compared in each time point.

Figure 7. Mean incremental change of plasma insulin concentrations in prediabetic men (n=15) with SEM; * indicate P<0.05, significance was compared in each time point.
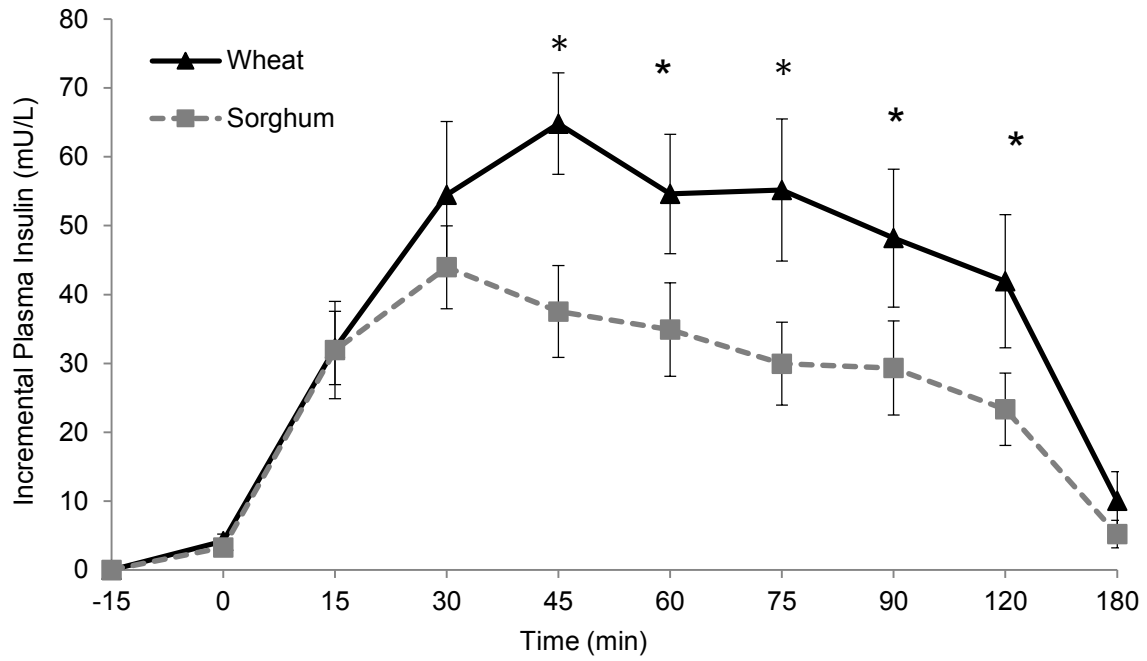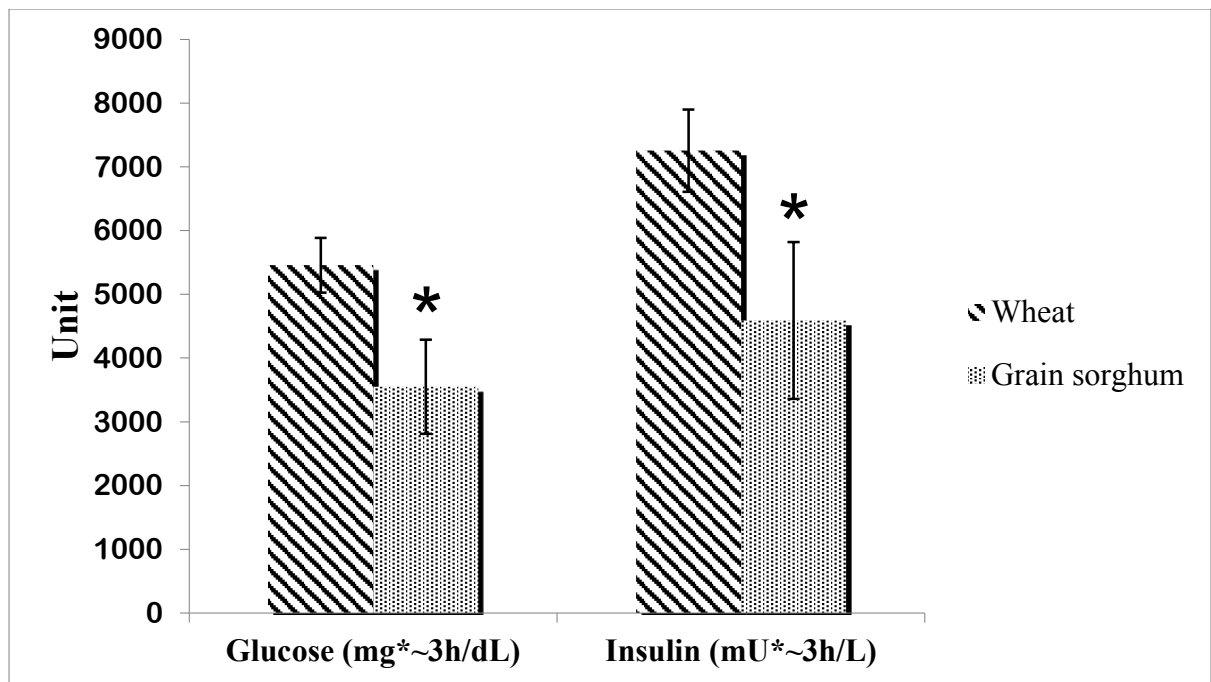
Figure 8. Total mean incremental area under the curve (iAUC) for plasma glucose and insulin concentrations in prediabetic men (n=15) with SEM; * indicate P<0.05 significance

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