EFFECTS OF DIGITARIA EXILIS (FONIO) ON

INFLAMMATION AND DIABETES

PATHOGENESIS

By

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INTRODUCTION

I.1 Context and Justifications

Diabetes and other chronic diseases used to belong to the western world. Currently, their incidence in the less developed countries is growing at a frightening rate. Excess consumption of energy-dense foods and considerable reduction of physical activities remain the main prevailing factors. In the past, farm work, field activities, long walks between villages, daily wrestling, staple foods essentially based on cereals such as millet (long sugar polymers), and scarcity of soft drinks contributed to preserve populations against chronic diseases. This phenomenon caused public health specialists to think that continents such as Africa were of minor concerns regarding chronic diseases (WHO, 2004).

Contrary to the past period, modern African societies are characterized by sophisticated transport systems, abundance of energy-dense foods (particularly in big urban cities), sedentary and European lifestyle. All these combined factors have contributed to the emergence of chronic diseases.

For instance, according to the World Health Organization (WHO) 2004 report, 143 000 Senegalese suffered from diabetes in year 2000, whereas by 2030, if no action is taken, there will be 421 thousand (a 3-fold increase). This rate of increase is higher than those observed for the world because in the year 2000, 171 million people were recorded to be stricken by the disease worldwide and this number is expected to rise to 366 millions in 2030 resulting in a 2.14 fold increase. As for those countries known to have high mortality and morbidity rates due to the disease, a WHO report estimates that there will be just a 1.7 and a 1.4 fold rise in the USA and France respectively, thanks to the energetic measures taken by these nations. Therefore, actions should be taken in order to tackle this overwhelming situation in Africa in general and Senegal in particular. Those actions should be achieved by way of an adequate and inexpensive nutrition therapy.

In developed countries, in addition to nutrition education, several drugs have already been developed for chronic diseases management. However, due to their high costs and lack of availability, most individuals from developing countries, especially those from Africa, cannot afford them and the long-term use of these drugs may be associated with detrimental side effects. Additionally, resources for nutrition education are limited.

The alternative is to develop inexpensive functional foods from local products that provide compounds with properties scientifically verified to be potent in the treatment of chronic diseases such as diabetes and its associated complications. Aside from their basic nutritional roles, functional foods can provide beneficial physiological effects against targeted diseases.

I.2 Goals

Long-term goal

Development of a functional food for diabetes care from fonio that was shown to have subsequent potential because of its high concentrations of beta-glucan, chromium, zinc, iron and important sulfur-containing amino acids (Traore 2004).

Short-term goals

(1) To investigate the possible modulatory properties of fonio and rice supplemented diets on inflammation and the pathogenesis of diabetes in KK/HIJ mice.

(2) To investigate the effects of fonio extracts on inflammatory cytokines in healthy non-diabetic CD1 mice.

(3) To investigate the mechanisms by which fonio extracts modulate the secretion of inflammatory cytokines.

1.3 Objectives

(1) Four-week old KK/HIJ transgenic male mice will be fed either the AIN 93 diet or the same diet fortified with 30% fonio or rice flours (n=20). Ten mice per dietary treatment group will be sacrificed after 3 or 4 months of feeding. Glucose and insulin tolerance tests (GTT & ITT) will be measured by standard techniques.

(2) Non diabetic healthy CD1 mice will be treated with bacterial lipopolisaccharides (LPS) in the presence and absence of fonio extracts.

(3) Monocytic THP1 cells will be treated without and with different concentrations of fonio extracts for 24-72 h in order to investigate the mechanisms by which fonio extracts modulate cytokine secretion.

1.4 Hypotheses

(1) Fonio, not rice, will significantly improve diabetic conditions in KK/HIJ mice. We define improved diabetic conditions as a decreased risk of developing insulin resistance, glucose tolerance, reduced weight gain, and down-regulation of the following pro-inflammatory cytokines: TNF-alpha; IL-6 and IL-1 beta. The anti inflammatory cytokine IL-10 is expected to be lower in the fonio-fed KK/HIJ group.

(2) The fonio extracts will induce in vivo and in vitro secretion of pro-inflammatory cytokines in the LPS-challenged CD1 mice.

(3) Fonio extracts will modulate the immune response of monocytic THP1 cells via their differentiation into macrophages.

CHAPTER II

REVIEW OF LITERATURE

II.I Diabetes

Definition

Several types of chronic diseases characterized by impaired glucose metabolism resulting in elevated amounts of blood glucose due to alteration in insulin production or insulin action or their combined effects. The main and most discussed diabetes are the type 1 and type 2 diabetes mellitus. They are different in their etiology, pathology and risk factors, therapy and evolution. Type 1 is an autoimmune condition which is present when the body creates self immunoglobulin to destroy its own pancreatic beta-cells. Although it can strike at any age, it mainly occurs in children and young adults (Dedoussis et al 2007; DeFronzo 2004; Hall and Davies 2008). Type 2 diabetes mellitus that is the focus of this present study starts its onset with the development of insulin resistance before reaching its final stage. Factors such as age, obesity, family history of diabetes or gestational diabetes, physical inactivity, race and ethnic group are risks that can prompt the disease.

Diagnosis of diabetes

The fasting plasma glucose (FPG) test. This is the first test administered in order to diagnose diabetes. it measures blood glucose in a person after an overnight fast of at least 8 hours. The test is suitable to detect both diabetes and pre-diabetes. A blood glucose level of 99 mg/dl or below is considered normal; Patients with values between 100 to 125 mg/dl are considered pre-diabetic or insulin resistant. Those that have values of 126 or

above are considered diabetic clients. All these tests need to be replicated in different days before diabetes is confirmed. Although FPG is very convenient and low cost, it does miss some diabetes or pre-diabetes that can be detected with OGTT. Therefore additional tests are needed:

OGTT. This is a more sensitive test especially for detecting pre-diabetes but invasive and less convenient. The OGTT is performed after fasting for at least 8 hours prior to. The plasma glucose levels are measured immediately before and 2 hours after a person is given a water solution containing 75 grams of glucose. People whose blood glucose levels range between 140 and 199 mg/dl 2 hours after, have pre-diabetes also called impaired glucose tolerance (IGT).

Having IGT, like having impaired fasting glucose (IFG), means that a person has not contracted diabetes type 2 but has an increased risk of developing it. But if a 2-hour glucose concentration of 200 mg/dl or above is detected and confirmed by repeating the oral glucose tolerance test on another day the person has diabetes. All these tests cited above (OGTT, FPG) require at least an eight-hour fasting period; $HbA1_C$ is a third test that is very precise in confirming the presence of diabetes and that doesn't require the fasting period (Hawkins and Rossetti 2005; De Belvis et al 2009).

$HbA1_C$. Glycated hemoglobin (HbA1c) is considered an established measure of glucose control in people living with diabetes and is a measure of the degree of exposure to circulating blood glucose over time (Nair et al, 2011). With the improvement of its standardization, there is renewed interest in the potential use of HbA1c as a valid diagnostic test of diabetes. Recently, the International Expert Committee (IEC) (2009) proposed an HbA1c level of $\geq 6.5\%$ to confirm the presence of diabetes, and $\geq 6.0\%$ for

diagnosing impaired glycemic states. And in 2010, American Diabetes Association (ADA) officially recognized HbA1c as a diagnostic test recommending cut-points of ≥6.5% for diabetes and 5.7–6.4% for high risk groups (American Association of Clinical Endocrinologists/American College of Endocrinology, 2010; HbA1c test results are also less variable than blood glucose measurements. The within-individual day-to-day variability is less than 2% for HbA1c, whereas variability with FPG ranges between 12–15% for FPG and 16.6% for OGTT. But there are some limitations to the HbA1c test due to some important limitations such as hyperbilirubinaemia, elevated serum triglycerides, pregnancy, age, genetic determinants (including race), pregnancy, malaria (Lapolla and colleagues 2011).

Random Plasma Glucose Test. According to the National Institute of Health (www.diabetes.niddk.nih.gov), blood glucose levels can be taken randomly. In this case, if values of 200 mg/dl or higher are found in people with the the following symptoms, it can be concluded that diabetes is present:

Polyuria (increased urination)

Polydipsia (increased thirst)

Unexplained weight loss

Other symptoms such as fatigue, blurred vision, increased hunger, and sores that do not heal will also be considered. Then the FPG test and/or OGTT are performed another day to confirm the diagnosis (www.diabetes.niddk.nih.gov).

Risks factors

Weight. Being overweight or obese is a primary risk factor for type 2 diabetes. Fatty acids levels are directly related with the development of insulin resistance (Mokdad et al 2003).

Fat distribution. If your body stores fat primarily in your abdomen, your risk of type 2 diabetes is greater than if your fat stores are located in your hips and thighs (Folsom et al 2000).

Inactivity/sedentarism. Low activity level increases chances to develop of diabetes type 2. Physical activity helps control weight. Ingested glucose and other starchy foods are used as energy (mostly ATP) and cells become more sensitive to insulin. Also low activity is strongly correlated to early death in people with diabetes type 2 (Colditz and Mariani 2000; Church et al 2004)

Family history. The risk of type 2 diabetes increases if your parent or sibling has type 2 diabetes (Bochud et al 2004; Barone et al 2008)

Race. Blacks, Hispanics, American Indians and Asian-Americans are more likely to develop type 2 diabetes than whites. Reasons are still unclear (Stuart et al 2000; Davidson and Schriger 2010).

Age. The risk of type 2 diabetes increases with age, especially after age 45. This is mainly due to a decrease in exercise that leads to a rapid loss of muscle mass with an increased weight gain with age. But the incidence of diabetes type 2 is also increasing dramatically among children, adolescents and younger adults (Fagot-Campagna et al 2005; Barone et al 2008).

Prediabetes/insulin resistance. As the blood glucose level rises after a meal, the pancreas releases insulin to help cells take in and use the glucose. Prediabetes is a condition in

which your blood sugar level is higher than normal, but not high enough to be classified as diabetes type 2. Left untreated, prediabetes often progresses to type 2 diabetes (DeFronzo, 2004). An impaired insulin secretion derives from several factors such as a defect in proinsulin biosynthesis, glucose toxicity, beta-cell dysfunction, and lipotoxicity (Kahn, 2001). Among all the above cited factors, beta cell dysfunction is considered a common trait in diabetes type 2 (Leahy 1990; Weyer et al 1999). Also beta cells dysfunction combined with fat accumulation lead to insulin resistance (Hawkins, 2005). Aggravation of insulin resistance interrupts normal physiologic actions of the hormone triggering the development of hyperinsulinemia that will eventually develop into diabetes type 2 and other conditions such as hypertension, dyslipidemia, atherosclerosis, and malignancy (Folsom et al, 2000; Calle et al, 1999).

High LDL/low HDL. Studies show that high levels of LDL combined to low levels of HDL led to the development of type 2 diabetes (ADA, 2008; Stumvoll et al, 2009).

Hypertension. Weycker and colleagues showed that all persons with hypertension, irrespective of age, sex, and BMI, had considerable risks of contracting diabetes. Among them, those that are overweight or obese are at substantially elevated risk of diabetes, regardless of age (Weycker et al, 2009).

Environmental factors/genetics. **Figure 1** describes how environmental factors directly interact with susceptibility genes into the development of type 2 diabetes or via obesity, and insulin resistance.
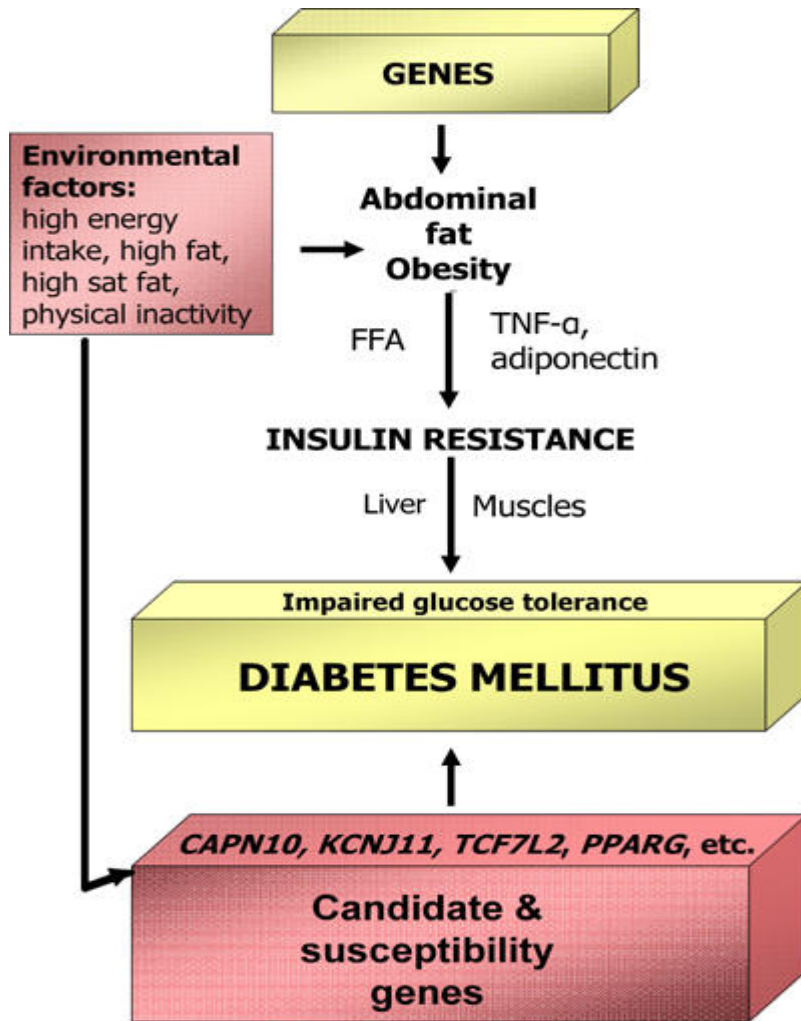
Figure1 Interrelation between genes and environmental factors in type 2 diabetes (Dedoussis et al, 2007. Rev Diabet Stud. 4(1):13-24. Epub)**.**

II.2. Cytokines

When challenged The immune system reacts by putting into play several white blood cell types, but the communication between those cells is mainly mediated by soluble factors recently known as cytokines (Gordon et al, 2008). Figure 2 (Munoz et al, 1995) describes the events leading to production of several cytokines when macrophages were challenged with lipopolysaccharide (LPS). During the last decade, more than 30 factors

have been purified with their primary structures described and their genes determined and cloned by recombinant DNA methods (Munoz et al, 1995). The body can recognize antigens and initiates the transcription of various cytokine genes that are translated in the cytosol and secreted into the extracellular space, and alike hormones they transmit their biological signals mostly by binding by high or loose affinity to specific ligands (cell surface receptors) thus triggering needed biological activities to protect targeted cells or organs. Apart from being pleiotropic and redundant in their actions, it is important to know that cytokines are mostly interdependent since the presence of another one or others can drastically change the direction of action of a given cytokine (for instance one cytokine that normally upregulates a biological action can downregulate it with the presence of another one [1]. The pro-inflammatory cytokines production is essential to a correct response to inflammatory status but an excessive production of them may be detrimental since resulting in most cases in an increase morbidity and mortality. Antioxidant defenses are exhausted that can lead to cells and tissue damage (Gordon et al, 2008).
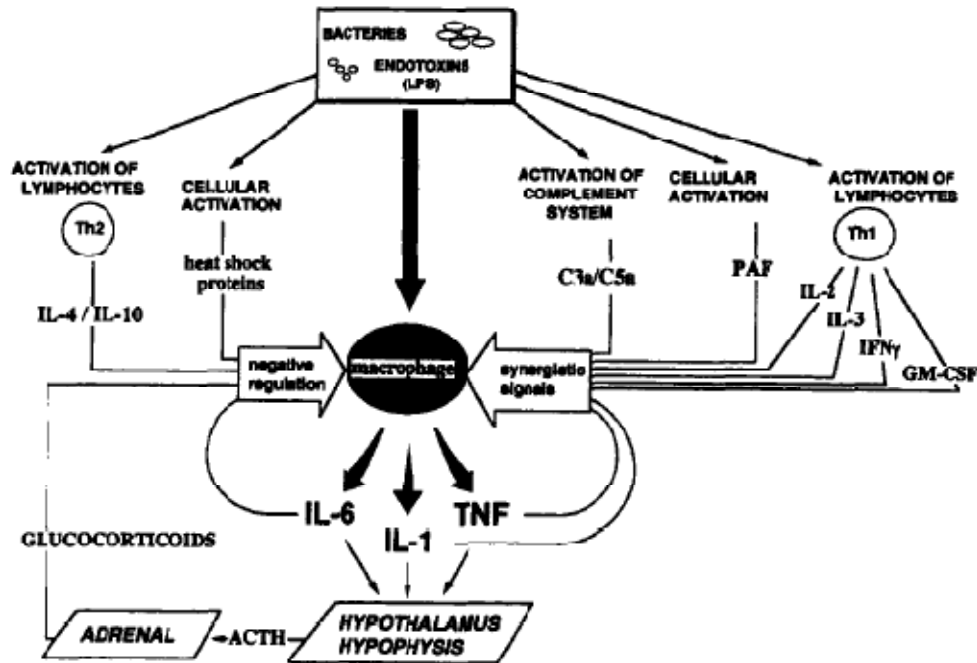
Figure 2. Positive and negative signals on cytokines production. Reproduced from the works of Munoz and his colleagues (Munoz et al, 1995).

Role of cytokines as therapeutics

Manipulation of cytokines has been a novel science and a tremendous breakthrough in the treatment of many diseases (Vilcek and Feldmann, 2004) that can clearly be separated in two distinct strategies. On the one hand, the over-expression of harmful cytokines are reduced or blocked by the administration of therapeutics, nutraceuticals or other dietary compounds and on the other hand, purified recombinant cytokines are administered to induce a positive physiological activity resulting in soothing or curing an ailment. Hematopoietic growth factors (colony stimulating factors) and interferons can be cited as successful cases in the treatment of diseases.

Characterized around 1950, cytokines are composed of regulatory proteins that are mainly produced by white blood cells and other cell types of humans and other animals. They have a wide range of actions among which the regulation of the immune system. In

1953, Bennett and Beeson demonstrated the existence of Interleukin 1 (IL-1) by treating

normal rabbits with either extracts of tissues and or polymorphonuclear leukocytes

collected from sterile peritoneal exudates in quantities of 200 to 600 million suspended in

sterile physiological saline. These peritoneal exudates were obtained from normal rabbits

that were challenged by *Serratia marcescens* endotoxin. The extracts of tissues did not

produce any effect but those of the polymorphonuclear leukocytes contained a heat-labile

substance or fever-producing agent that produced fever in the febrile animals that

reflected inflammation. Then, for the first time, Il-1 was evidenced and named as pyrogen

(Bennett and Beeson, 1953). Also, the supernatants fluids obtained from the original

peritoneal exudates exerted the same fever-producing activity. They concluded that the

polymorphonuclear leukocytes that were producing fever in the rabbits were identical to

those that would proliferate, produce heat and dye during acute injury. But carefully, they

stated that other cells that appeared to be inactive should not be excluded from being

capable of producing fever in different conditions or amounts.

Since then many studies were conducted on how IL-1 were involved in the pathogenesis

of several diseases. Recently, an IL-1 inhibitor in the form of a soluble IL-1 receptor

antagonist was approved for therapeutic use against rheumatoid arthritis. The mechanism

of action involved is the prevention of active IL-1 from its receptor. The researchers

recruited a total of 412 RA patients (18-75 of age) from centers of 11 European countries

that were randomized into 4 groups: placebo (n = 121) or IL-1Ra at a daily dosage of 30

mg (n = 119), 75 mg (n = 116), or 150 mg (n = 116). Two methods (Genan and Larsen

erosive joint count) of hand radiographs were performed at baseline, 24 weeks, and 48

weeks and scores were recorded. Significant reductions in the score for progression of

joint space narrowing (JSN) of 58% and a decrease in erosion (38%) and a combined

reduction of 47% of JSN and erosion as total score were observed in all treatment groups

at week 24. IL-1Ra reduced radiologic progression of RA. Both methods used strongly

correlated for each individual time point from baseline (0.83 with $P < 0.0001$ for both

methods), but much less strongly for assessments of disease progression between week

24 to week 48 (0.36 and 0.41 ($P < 0.0001$ respectively for the Genan and Larsen

methods).

Several studies indicate that pro-inflammatory cytokines may play roles in eating

disorders. The pro-inflammatory cytokines such as IL-1, IL-6, TNFα are known to

decrease food intake. They mediate infection and inflammation during the anorexia as

part of a of a sickness response (Wong and Pinkney, 2004). These cytokines are believed

to influence the energy balance by their actions on the appetite-regulating pathways of

the brain; they can follow three different itineraries to reach the neural targets: via the

vagal afferents route, or by direct contact with the cerebral epithelium or reaching the

brain by passive diffusion in regions that are not tightly covered by the blood brain

barrier. Deregulation of the cytokine network may be the primary cause for medical

complications in patients with anorexia who suffer from chronic underweight (Wong and

Pinkney, 2004).

Similarly, Dantzer examined whether an increase in local proinflammatory cytokines

would be associated with increased negative mood and decreased positive mood. For that

purpose,189 healthy adults were recruited and exposed to rhinovirus or influenza virus

during a 6-day period of quarantine and the outcome variables at baseline and on each of

the 5 post-challenge quarantine days were infection, objective signs of illness, nasal IL-

1β, IL-6, and TNF-α, and self-reported affect. In the 153 persons who became infected following exposure to the challenge virus, daily production of the three cytokines, was associated with reduced concurrent daily positive affect. Findings support a causal association between pathogen-induced local cytokine production and changes in positive affect over a 24-hour time line. Therefore, IL-1, IL-6, and TNF-α, were not only responsible for the local inflammatory response, but in addition, they synchronize the physiological and behavioral components of the systemic acute phase response to infection. In addition, they induced a behavioral pattern referred to as sickness behavior, including anorexia, psychomotor retardation, sleep disturbances, pain, anergy, and anhedonia that is the inability to experience pleasure [2].

Role of cytokines in diabetes and chronic diseases

Tuttle and coworkers showed that the pro-inflammatory cytokines IL-6 and TNF-alpha were chronically increased among type 2 diabetic women with or without cardiovascular diseases (CVD) compared to non-diabetic women. They also reported that inflammation was involved in diabetes pathogenesis (Tuttle et al, 2003). Beta-cell apoptosis was reported to be mediated by nitric oxide (NO) presence and oxygen-derived free radicals; the beta-cells under stress express very low levels of free radical scavenging enzymes (superoxide dismutase and catalaze) leading to increased suceptibility to free radical-induced beta-cell damage (Kawasaki, 2004). Also IL-1 alone or associated with INF-gamma led to the beta-cell synthesis of NO and other cytokines to trigger cell apoptosis via the expression of NO synthase (Kawasaki, 2004).

Obesity, Inflammation and diabetes

Not long ago, **TNF-alpha** was identified as the first molecular link between inflammation and obesity. This inflammatory cytokine was found to be over-expressed in the adipose tissues of rodent models of obesity (Hotamisligil et al, 1993; Sethi and Hotamisligil, 1993)

Recently, many studies investigated how cells behave under the stresses of obesity. Major breakthroughs had been done in the understanding of the intracellular signaling pathways activated by inflammatory and stress responses and how these pathways influence and inhibit insulin signaling.

Insulin initiates cells response through binding to its receptor on the surface of insulin-responsive cells. This binding stimulates an auto-phosphorylation of the insulin receptor that triggers the phosphorylation of several substrates, including members of the insulin receptor substrate (IRS) (White, 1997; Saltiel and Pessin, 2002). Exposure of cells to **TNF-alpha** and/or high levels of free fatty acids blocks the phosphorylation of serine residues of IRS-1 (Yin et al, 1198; Aguirre et al, 2000). Without phosphorylation of the serine residues, association of IRS-1 with the insulin receptor is not allowed. Therefore, the downstream signaling is practically shut down, and consequently insulin action is drastically reduced. (Paz et al, 1997; Aguirre et al, 2002). This is a primary mechanism through which inflammatory signaling (via TNF-alpha) leads to insulin resistance.

Nutrition and Inflammation

Nutrition status plays a major role in infection. People with a good health and nutritional status respond by producing enough inflammatory cytokines when they experience infection. Whenever the immune system gets rid of the infection, the inflammatory

cytokines are cleared (Gordon et al, 2008). However when malnutrition is present, the body doesn't produce enough inflammatory cytokines in case of infection and these populations become highly sensitive to infectious diseases (Neumann et al, 1975).

Protein-energy malnutrition (PEM). Studies have shown that it is the loss of lean (protein) rather than fat tissue that has the most critical clinical impact. For instance, Neumann and colleagues (1975) studied the immunological response of normal and malnourished Ghanaian children; and they evidenced for the first time, that cytokine production was impaired in protein-energy malnutrition (PEM). Results of their study showed that malnourished children had four times the amount of infection and a 20% increase in parasitic infestation rates as compared to normal children. Also they (PEM) had lower circulating concentrations of the acute phase protein and C3 complement.

Table 1: Cytokine Production in protein-calorie malnourished subjects (from Neumann et al, 1975)

Cytokine Production in Protein-calorie Malnourished Subjects

Patients	Induction	Assay	Cytokine Level	Reference
Adults	Zymosan-stimulated leukocytes	indirect biological determination	↓ LEM	Keenan et al. Ref. 28
Adults	LPS-stimulated monocytes	indirect biological determination	↓ LEM	Hoffman-Goetz et al. Ref. 29
Adults	S.epidermis stimulated monocytes	indirect biological determination	↓ EP synthesis	Kauffman et al. Ref. 30
Pre-school children	LPS-stimulated monocytes	bioassay for thymocyte proliferation	↓ IL-1	Bhaskaram et al. Ref. 31
Infants	LPS-stimulated mononuclear cells	ELISA	↓ IL-1 and TNF-α	Muñoz et al. Ref. 32
Infants	LPS-stimulated whole blood cells	ELISA	↓ TNF-α and IL-6	Doherty et al. Ref. 39

LEM = leukocyte endogenous mediator; EP = endogenous pyrogen

Later on, Hoffman-Goetz and his colleagues (1981) reported that leukocytes from patients suffering from PEM on admission produced low levels of interleukin-1 alpha (IL-1alpha) after being challenged with LPS. After nutritional rehabilitation (via total parenteral nutrition) , their response became normal.

Lipids modulate the immune system by altering membrane fluidity, producing lipid peroxides, regulating eicosanoid metabolites and interacting with cellular activation processes.

Lipids. Epidemiological studies in populations of coastal Eskimo, Japanese and Dutch subjects have shown that a high intake of n-3 fatty acids correlates with a low incidence

of cardiovascular and inflammatory diseases such as asthma and Type I diabetes mellitus Siess et al, 1980; Strasser 1985, Kromhout 1990).

The above findings are recently explained by the fact that sea foods are rich in n-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (22:6 n-3); and they are very low in arachidonic acid (20:4 n-6) [Jump, 2002]. EPA and arachidonic acid are further metabolized to eicosanoids and eventually to prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) via the cyclooxygenase (PG, TX) and lipoxygenase pathways (Jump, 2002). Eicosanoids have several physiological functions including: mediation of inflammation, and aggregatory and vasoconstrictory effects (. Eicosanoids derived from n-3 fatty acids are anti or less proinflammatory (PGE3, LTB5). EPA-derived PG inhibit TXB2-mediated platelet aggregation and promote vasodilation derived from arachidonic (n-6) acid are pro-inflammatory (prostaglandin E2, PGE2; leukotriene B4, LTB4), potent vasoconstrictor and platelet activator (TXB2) [

II.3. Digitaria exilis (fonio)

Fonio is a variety of millet mainly grown in West Africa, consisting of tiny kernels with a size approximating 700 μm of diameter. According to the geographic location, it is given different names such as "Acha" or "Fundi"; its English name is "hungry rice" (Sartelet, 1995).

Habitat

According to National Academy of Sciences (1996), fonio is probably the oldest African cereal and was being cultivated across the arid savannas. This cereal in fact was used as the major staple food. Nowadays the crop still remains important in areas scattered from

Cape Verde to Lake Chad. In certain rural regions of Mali, Burkina Faso, Guinea and Nigeria, it is still heavily consumed but the incidence of diabetes in these particular regions is not yet documented. Compared to the other cereals, fonio has received little attention due to the fact that scientists and other decision makers have misunderstood the plant. This neglect, characterized by few publications, was brought about by English colonialists who knew little about the cereal and called it "hungry rice", a misleading term. In fact local populations harvested fonio not because of hunger but because they liked its taste (National Academy of Sciences, 1996); and because of its ability to withstand infertile soils and low rainfalls in the Sahelian regions (Sartelet, 1995).

In some places fonio is particularly reserved for chiefs, royalty and special occasions such as ancestor worship. Harlan (1993) reported that according to the Dogon tribe in Mali, the whole universe emerged from a seed of fonio. Harlan made the following statement on fonio: "First, let me call attention to some of the other cereals, both wild and tame. Fonio or acha, *Digitaria exilis*, has been given the misnomer "hungry rice" by English colonials. It is not grown to relieve hunger but because of its quality. It is a chief's food, a gourmet item, and couscous made of fonio is better than couscous made from wheat''.

Biochemical properties

Fonio is reported to be different from other cereals in that it displays the lowest amino acid pattern but offers the singularity of being much richer in methionine and cysteine (sulfur-containing amino acids) and leucine whose concentrations are higher than those defining the FAO reference protein (Sartelet et al, 1996; Thompson et al 1993; Becker et al, 1986).

Also, fonio has less flavonoid contents when compared to the other cereals with 500 mg/kg against 1 to 1.2 g /kg from Pennisetum millet and Pennisetum americanum. But in the latter cereals, the types of flavonoids are composed of C-glucosyl derivatives (less goitrogenic); fonio once again differs from those species by containing two aglycones (150 mg of apigenin and 350 mg of luteolin) that are approximately seven times more potent than C-glucosyl derivatives (Sartelet et al, 1996). This feature implies that the lower content of flavonoids in fonio is compensated by the presence of the most active ones. In addition, contrary to the millet species, fonio did not contain thioglucosides or cyanogenic glucosides that are transformed after hydrolysis or bioconversion into thiocyanates known to be goitrogenic compounds (Van Etten et al, 1973; Delange et al, 1976).

In their study, Sartelet and his colleagues showed that luteolin from Fonio showed a regulatory effect on thyroid functions of pig thyroid cells. First, they demonstrated that both luteolin and apigenin had inhibitory effects on thyroid hormone production by impairing thyroid peroxidase activity; however, luteolin displayed a highly stimulatory effect on thyrotropes on day 4 of treatment, through deactivation of cAMP phosphodiesterase, thus allowing a highly significant overproduction of the second

messenger cAMP nucleotide that is dependent on the thyrotropin-stimulating hormone (TSH) action on thyrotropes. But the net result of these opposing effects was slightly goitrogenic.

They concluded that further studies should be conducted to better elucidate the role, function and mechanism of action of the fonio aglycones on thyroid hormones (Sartelet et al, 1996).

Nutritional aspects.

The proximate composition of white fonio is comparable to wheat with the husked grain containing 8 percent protein and 1 percent fat. In some black fonio samples, the protein content was 11.8 (National Academy of Sciences, 1996). Becker and later on De Thompson showed that the difference with the other cereals was the amount of the essential amino acids, especially the sulfur containing ones: approximately 7.3-8.0 % of the amino acids in fonio protein are methionine and cysteine. The amino acid profile compared to that of whole-egg protein showed that except for the low score for lysine 46%, the other scores were high: 72 for isoleucine, almost 100 for valine, tryptophan, threonine and phenylalanine; 127 for leucine; 175 for total sulfur and 189 percent for methionine (De Thompson et al 1993; Becker et al, 1986). These figures show the important potential of Fonio, not in terms of a survival crop but as a standard diet (De Thompson et al 1993; Becker et al, 1986). In addition, a study in 2004 (Traore, 2004) showed that fonio was rich in beta-glucan and chromium, two compounds known to display important beneficial effects on diabetes. Beta-glucan has been extensively studied and reviewed for its alleged properties to deal with chronic diseases (Volman et al. 2008; Brown and Gordon, 2003; Burkitt et al 1981). The importance of fiber, beta-glucan,

chromium the sulfur containing amino acids and their interaction with diabetes will be further reviewed.

II.4 Effects of fonio active compounds on diabetes

As previously mentioned, late studies on fonio revealed that the cereal was rich in dietary fibers containing subsequent quantities of beta-glucan. In addition the cereal displayed important amounts of chromium and sulfur-containing amino acids.

As early as 1960, has there been an ongoing growing interest in the study of the positive health effects of dietary fibers. Researchers have so far succeeded in mapping out their molecular components, molecular weights, and physical properties in order to have a better understanding of their functions in the human body. Both human and animal models have been used to directly assess beta-glucan effects and their mode of actions. One of the important components of dietary fibers that investigators thought to be among the more potent was beta-glucan, a soluble dietary fiber present in some cereals and some mushrooms. Beta-glucans have been extensively studied; and most of the related beneficial health effects from dietary fibers have been associated with them. Some works on beta-glucan confined themselves to their prebiotic properties; others, to their capability of lowering blood glucose and potentiating insulin action or to their potential of boosting the immune system and consequently, their capability to fight against certain chronic diseases that impair the immune system (diabetes, hypertension, cardiovascular diseases and cancer).

Several models have been proposed to explain how they interact with the human body. Some mechanical effects have been clearly defined when dietary fibers bind cholesterol in the gut to excrete it in the feces and not allowing its re-uptake via the enterohepatic

pathway circulation. But it is not long ago that researchers really started to break through beta-glucan functions at the molecular level. Also, chromium was known as a co-factor of insulin that would potentiate ita action on glucose and fat metabolism, but recently several studies showed that the tracemineral was involved in several inflammatory processes. As for sulfur-containing amino acids, there is a paucity of data or studies relating them directely their effects on diabetes and its complications. But the effects of other sulfur-containig compounds on diabetes have been extensively investigated. The objective of this review is to start with a brief presentation of the chemical structures and characteristics of these selected compounds, then give an overview of their metabolism, their role against chronic diseases in general and diabetes in particular in order to further discuss their immune modulating properties.

Chemical structure of β-Glucans

β-Glucans are composed of several linked glucose molecules, which are major structural components of the cell walls of yeast, fungi and some bacteria (Figure 1). Also cereals, such as barley and oat contain β-glucans as part of their endosperm cell walls. Depending on the source, there are clear differences in macromolecular structure between β-glucans (Figure 2). The cell wall β-glucans of yeast and fungi consists of 1,3 β-linked glycopyranosyl residues with small numbers of 1,6 β-linked branches. In contrast, cereals (oat and barley) cell walls contain unbranched β-glucans with 1,3 and 1,4 β-linked glycopyranosyl residues, whereas β-glucans from bacterial origin are unbranched 1,3 β-linked glycopyranosyl residues (Volman et al. 2008). Furthermore, besides differences in type of linkage and branching, β-glucans can vary in solubility, molecular mass, tertiary structure, degree of branching, polymer charge and solution conformation (triple or single

helix or random coil). All these characteristics may influence their immune modulating effects. For example, Brown and Gordon (2003) have recently suggested that high molecular weight (MW) and/or particulate β-glucans from fungi directly activate leukocytes, while low MW β-glucans from fungi only modulate the response of cells when they are stimulated with cytokines. With respect to the characteristics of the β-glucans, it should be noted that the isolation method may influence these characteristics. Consequently, differences can be expected between various β-glucans differentially isolated from the same source (Volman et al. 2008).

Physiological effects of β-glucans

It has been suggested that diabetes mellitus is highly correlated with the fiber-depleted, highly-refined carbohydrate diet of the "civilized man" (Burkitt 1981). Population studies, as well as clinical and experimental research showed diabetes as being one of the diseases most clearly related to inadequate dietary fiber intake (Burkitt et al 1981, Vahouny et al 1982). These results indicate that while the intake of refined sugars should be curtailed, the intake of complex carbohydrate sources that are rich in fiber should be increased.

The term "dietary fiber" refers to the components of the plant cell wall as well the indigestible residues from plant foods. The types of fiber that exert the most beneficial effects on blood sugar control are the water-soluble forms. Included in this class are hemicelluloses, mucilage, gums, and pectin substances. As early as 1981, one study (Simpson et al 1981) showed that these types of fibers were capable of slowing the digestion and absorption of carbohydrates, thereby preventing rapid rises in blood glucose; increasing the sensitivity of tissue cells to insulin, thus preventing the excessive

secretion of insulin; and improving uptake of glucose by the liver and other tissues, therefore preventing a sustained elevation in blood sugar.

In the study cited earlier, a high carbohydrate, legume rich, high fiber diet improved all aspects of diabetic control. Other studies showed that when diabetic patients were supplemented with 14 and 26 grams of guar gum per day, they required less insulin and had less glycosuria (Vahouny et al 1982, Jenkins, 1980).

Both crude fibers and total dietary fibers contribute to the lowering of the glycemic index of foods (Jenkins et al, 2002) and are inversely related to body mass index (Mckeown et al 2002), HbA1c levels and ketoacidosis risk (Toeller 2002). The glycemic index concept, an extension of the fiber hypothesis, suggests that fiber consumption reduces the rate of nutrient influx from the gut, and as a result significantly improves glycemic control in diabetic clients. Also, fiber consumption reduces urinary C-peptide excretion in healthy people as well as circulating insulin and serum lipids in hyperlipidemic subjects (Jenkins et al, 2002; Giacco et al, 2002). And consumption of high fiber diets has been associated with higher HDL-cholesterol concentrations in cohort studies, with decreased risk of developing diabetes and cardiovascular diseases (Kumar et al 2002; Simin 2002; McKeown et al 2002; Mujumdar, 1995).

Viscosity and molecular weight as physiological determinants

The physiological functions of dietary fiber are often attributed to their physico-chemical properties: water holding capacity, swelling, diffusion-suppressing ability (through viscosity enhancement and gel-formation), binding properties, and susceptibility or resistance to bacterial degradation and fermentation (Dikeman and Fahey 2006). The mechanisms by which a soluble fiber, such as β-glucan, exerts hypocholesterolemic and

hypoglycemic effects are still debated but the most common hypothesis is based on increased lumen viscosity (Battilana 2001 et al., Dikeman and Fahey 2006). It has been suggested that cereal β-glucans decrease the absorption and reabsorption of cholesterol, bile acids, and their metabolites by increasing the viscosity of the gastro-intestinal tract contents as well as delaying gastric emptying and the intestinal absorption of nutrients, such as digestible carbohydrates, and thereby reducing post-prandial hyperglycemia and insulin secretion. The latter have health benefits for those with type-2 diabetes, and are also associated with reduced risk of developing the disease and insulin insensitivity. Carbohydrate and lipid metabolism are closely interrelated and insulin has also been reported to increase hepatic cholesterol synthesis. Therefore, if fiber decreases carbohydrate absorption and insulin secretion, it may also contribute indirectly to the hypocholesterolemic effects (Dikeman and Fahey 2006, Wood et al. 2002). Bell et al., 1999 S. Bell, V.M. Goldman, B.R. Bistrian, A.H. Arnold, G. Ostroff and A. Forse, Effect of β-glucan from oats and yeast on serum lipids, Critical Reviews in Food Science and Nutrition (1999), pp. 189–202. Abstract | View Record in Scopus Cited By in Scopus (36)Furthermore, the increased viscosity caused by soluble dietary fiber influences fat emulsification by increasing emulsion droplet size which may impair fat absorption (Pasquier et al. 1996). However, Battilana and his colleagues (2001) found that the administration of frequent meals with or without β-glucans resulted in similar carbohydrate and lipid metabolism, whereas ingestion of a single meal containing β-glucan lowered post-prandial glucose concentrations. This might suggest that the beneficial action of β-glucans is mainly due to delayed and reduced carbohydrate absorption from the gut and does not result from the effects of metabolites produced by

fermentation of β-glucans in the colon. It is also likely that β-glucans do not only decrease the post-prandial glucose response due to high viscosity in the gastro-intestinal tract, but also reduce starch digestion by α-amylase. Symons and Brennan (2004) found that inclusion of 5% of a β-glucan-rich fraction in bread resulted in a significant decrease in the release rate of reducing sugars following the in vitro digestion of bread with pepsin and α-amylase. A study with ileostomy subjects showed that bile acid excretion increased by a mean of >50% in a diet with oat bran bread, compared with a similar diet in which 70% of the β-glucan was degraded by β-glucanase. This indicates that the high molecular weight β-glucan mediates increased excretion of bile acids: whole micelles or bile acids may become entrapped or encapsulated in the highly viscous β-glucan matrix, and then excreted from the small bowel or the viscous environment to reduce the formation of mixed micelles in the small intestine and/or decrease the reabsorption of bile acids in the terminal ileum (Lia et al. 1995).

Furthermore, a dependence of the hypocholesterolemic action on extractability, viscosity, pseudo-plastic flow behavior, hydrodynamic properties, and molecular weight of β-glucans was found in rats fed on diets based on various oat bran concentrates, supporting a mechanism based on high lumen viscosity (Malkki et al, 1992). A difference in the mechanism of physiological effects has also been observed between cereal β-glucans differing in their molecular features. Wilson and his colleagues (2004) demonstrated that the cholesterol-lowering activity of barley β-glucan in hamsters occurred with both low (175 kDa) and high molecular weight (1000 kDa) preparations. Several other animal studies of diets supplemented with cereal fiber or cereal fractions showed that the content of β-glucans and particularly that of soluble β-glucans, the molecular weight of the β-

glucans and endogenous β-glucanase activity, all of which affect extract viscosity, appeared to be strong predictors of the serum cholesterol response (Kahlon 1993 et al., Newman and Newman, 1991; Newman et al, 1992) . In a meta-analysis report, Brown et al (1999) identified 25 clinical studies involving a total of 1600 healthy, hyperlipidemic and diabetic individuals in the age range of 26–61. Daily doses of between 2 and 10 g of oat soluble fibers gave significant mean reductions in the total (–0.04 mmol/L/g of fibre) and low-density-lipoprotein (LDL, –0.037 mmol/L/g of fibre) cholesterol levels. It is generally considered that the cholesterol-lowering effect is larger in subjects with higher cholesterol levels, whereas the dose–response effect is not always clear.

Overall, the findings from many human studies reveal a substantial variation in the responses between the different trials and types of subjects studied. In addition to variation in the design (intervention) of the clinical trial (i.e. number and type of subjects involved, daily intake and number of servings, length of treatment, etc.), variation is also observed in the physiological responses of individuals. Also, in some cases, inconsistent results may be related to factors that influence the viscosity development by β-glucans, which is in turn determined by the molecular weight and extent of solubilization of the polysaccharide from the food matrix.

According to Anderson et al. (1990) and Mallki and Virtanen (2001), soluble fibers can affect satiety and promote weight loss for a number of reasons including a slower rate of meal intake, a delay in gastric emptying, moderate responses of plasma glucose levels and insulin secretion by the pancreas (insulin stimulates hunger), elevation of cholecystokinin (a gut hormone correlated with prolonged satiety) and production of gas and short-chain fatty acids by fermentation of the fibers in the colon.

A gelling β-glucan from barley (Glucagel) can cause a number of effects on the mammalian immune system, including upregulation of the release of the cytokine interleukin 1β (IL-1β) (indicating enhanced monocyte differentiation), changes to wound healing processes and enhancement of gut mucin secretion (Porter et al. 2006). Furthermore, the in vitro secretion of IL-1β, which is one of the early responses of the immune system to infection, increased as the dose and molecular weight of the β-glucan were increasing.

Effects of β-glucans on food matrix

The viscosity effects of β-glucans are often modulated by formulation and food processing and storage protocols. The dose–response of oat gum on plasma glucose and insulin levels of healthy humans consuming oat gum in a drink (in the range of 1.8–14.5 g after an oral glucose load of 50 g) has been studied (Wood 1994). Increasing the dose of oat gum successively reduced the plasma glucose and insulin responses relative to a control drink without gum, reaching a plateau dose–response at an intake of 6 g of β-glucan (the maximum effective dose). In a recent study by Cavallero et al. (2002), a linear decrease in the glycemic index of non-diabetic humans was found with increasing consumption of barley β-glucan in bread, the GI (glycemic index) being related to the total β-glucan content (TBG).

Digestion and metabolism of β-glucans

When ingested, beta-glucan starts to absorb water and swell. The rate of swelling and dissolving depends on the particle size and previous hydrothermal treatments. Increased volumes cause a distension of stomach affecting satiety. Data on the effect on gastric emptying are in part controversial. A concept often referred is that viscous dietary fibers

reduce the rate of gastric emptying, whereas coarse particles leave the stomach sooner (Wilmshurst and Crawley 1980). Only 0.16 or 0.13 of the beta-glucan given in the form of rolled oats or oat bran, respectively, was dissolved 1 h after feeding, and was transferred into the duodenum (Wilmshurst and Crawley 1980).

In addition to the effect of viscosity as such, a possible mechanism for the retardation of gastric emptying is the effect of products of colon fermentation. When non digested carbohydrates enter the proximal colon, they are fermented to short-chain fatty acids (SCFA), which decrease the gastric tone (Ropert et al. 1996).

Small intestine. As the mammalian enzymes are unable to hydrolyse beta-glucan, it remains nearly intact in the small intestine of healthy man and thus increases the viscosity throughout the small intestine. The increased viscosity caused by viscous polysaccharides has the following effects on the digestion and absorption:

- Hindered mixing of luminal contents may retard transport of digestive enzymes to their substrates (Schneeman and Gallaher 1985).

- Emulsification of lipids is impaired, partly due to decreased mechanical mixing caused by the reduction of the contraction movements (Edwards et al. 1988).

- Reduced absorption of nutrients due to retarded transport to the absorbing surface and to the increased thickness of the unstirred layer on the absorbing surface (Edwards et al.1988).

The retarded absorption of nutrients has been often ascribed to reduced diffusion rates. However, model experiments with guar gum (Edwards et al. 1988) have shown that it is

mainly due to increased resistance to contraction movements, and thus decreased mass transfer by convection.

Lipid digestion and absorption in the intestine. As shown in an in vitro study (Pasquier et al. 1996), viscous fibers affect the emulsification of lipids by causing an increase in lipid droplet size and thus a decrease in the contact surface between the lipid and aqueous phase. A study of ileostomic patients (Lia 1997) showed that oat bran in the test meal delayed the micellar lipid solubilization process and consequently reduced the secretion of chylomicrons into the circulation. In an earlier study by the same group (Dubois et al. 1995), it was found that 3 h after a meal containing 40 g oat bran, corresponding to about 5 g of soluble oat fiber, the level of the large-sized triglyceride rich lipoprotein fraction of blood was higher than the control meal 3 hours later. This increase was still higher after the subjects had been adapted to the oat bran diet for 14 days. In a number of other clinical studies, oat bran or oat gum has been found to reduce lipid absorption.

Mechanism underlying the effect of β-glucans on the immune system

After oral administration, β-glucans come in contact with the mucosal immune system (Figure 4). The intestinal epithelial cells together with the immune cells of the Peyer's patches play an important role in regulating immune responses. Oral administration of β-glucans can modulate the mucosal immune response by cells of the Peyer's patches as well as intestinal intraepithelial lymphocytes (Tsukada et al. 2003). It has been suggested that the protective effects of orally administered 1,3 β-glucans are mediated through receptor-mediated interactions with microfold cells-specialized (Figure 5) epithelial cells for the transport of macromolecules-in the Peyer's patches, which lead to increased cytokine production and enhanced resistance to infection.

Effects of various β-glucans have been examined in vitro and in several animal models, while only a few human studies have been carried out. Three clinical studies demonstrated that pre-treatment of high-risk surgical patients with poly-[1-6]--D-glucopyranosyl-[1-3]--D-glucopyranose glucan (PGG-glucan) supplied intravenously (Abbas & Lichtman 2003) decreased the infection incidence and need for antibiotics, shortened intensive care unit length stay, and ultimately improved survival compared to a saline placebo injection (Babineau et al. 1994, Babineau et al.1994). Only 3 days after trauma serum IL-1 concentrations were elevated in β-glucan treated trauma patients as compared to control treated patients, while TNFα concentrations remained unchanged (Browder et al. 1990). Similarly, Lehne et al. (2006) found no difference in cytokine and immunoglobulin concentrations in the blood of traumatized humans after 5 days treatment with three different concentrations of yeast β-glucan compared to baseline, although IgA concentrations were increased in saliva.

Furthermore, the finding that clinical parameters are improved without a severe elevation in concentrations of circulating cytokines and immunoglobulins suggests an immune regulatory function of β-glucans that goes further than simply immune activation, as they may prime instead of activate leukocytes in vivo to combat invading pathogens.

β-glucans as prebiotics and their impact on cancer treatment

Pigs fed with oat bran (mainly composed of beta-glucan) showed dramatic dose-dependent increases in colonic microbial load according to the concentration of the beta-glucan inside the roll or bran. The highest beta-glucan-content bran induced the highest counts in the large intestine from an initial level of about 10^6/g at the ileo-caecal junction, to about 10^{11}/g at the rectum. Increased microbial cell growth leads to an increased

binding of nitrogen and its excretion in feces (Knudsen et al. 1991) thus reducing the

nitrogen load to be excreted via kidneys. Furthermore, besides alleviating the kidney

burden, the rapid fermentation of soluble fibers in the large intestine by the human

natural flora, triggers interesting effects on colonic epithelium and colonic cells growth

and vitality that use the main byproducts (short-chain fatty acids) of beta-glucan

fermentation as an energy source. Among the short-chain fatty acids (SCFA) formed,

butyric acid unmistakably displays the most important effects in the colon. The colonic

mucosa may use butyrate as a preferential energy source (Roediger et al. 1982), and

butyrate is almost entirely cleared by the colonic epithelium (Bugaut and Bentejac 1993).

These acids stimulate cell proliferation in normal colonic epithelium, but retard the

growth of carcinoma cell lines (Kim et al. 1980).

Butyrate also has a differentiating effect on isolated cancer cell lines. Butyrate and also

acetate and propionate (Hague et al. 1995) induce apoptosis (programmed cell death) in

cultured colonic adenoma and carcinoma cells. The numerous mechanisms of action of

butyric acid on the colonic epithelium (Scheppach et al. 1998) and at the level of gene

expression (Smith et al. 1998) have been extensively studied. In addition, beta-glucan

may have a strong potential regulatory effect on glucose metabolism and insulin action

(Jenkins et al, 2002). In addition, beta-glucan was shown to lower efficiently LDL

concentration and the reference dose of a daily intake was 3 % from oat bran (Plotnikoff

and Infanger, 2000); and the study mentioned that the effect was dose-response

dependent.

II.5 Chromium.

Chromium in glucose and fat metabolism

Many studies have shown that this trace element is vital to proper blood sugar control. The studies showed that it works closely with insulin in facilitating the uptake of glucose into cells. Without chromium, insulin action is blocked and glucose levels are elevated (Anderson, 1992; Mooradian et al 1994). In some clinical studies, supplementing the diet with chromium has been shown to decrease fasting glucose levels, improve glucose tolerance and lower insulin levels and decrease total cholesterol and triglyceride levels while increasing HDL-cholesterol levels (Anderson 1992; Mooradian et al 1994). Most recently, Vincent and his colleagues had made a major breakthrough regarding the structure and role of chromodulin or low-molecular-weight-chromium-binding substance. They showed that the oligopeptide, composed of residues of glycine, cystein, glutamate and aspartate binds 4 atoms of chromium. The resulting holo form can potentiate 7 times the action of insulin without increasing its concentration by binding the insulin receptor-kinase; and interestingly, insulin stimulation was directly dependent on the number of bound chromium atoms (Vincent, 1999; Vincent, 2000).

Anti-inflammatory effects of chromium

Stress due to inflammation is very important among the obese and diabetic patients (Hilary Turtle, 2003). Jain and Krishnaswamy demonstrated for the first time that chromium chloride inhibited oxidative stress and TNF-alpha in U-937 monocytes (cell culture); according to the authors, TNF-alpha was associated with decreased insulin sensitivity in vivo in diabetes (Jain and Krishnaswarmy, 2001). Furthermore, Jain and coworkers showed that the reducing effect of inflammation by 17 beta-estradiol via

inhibition of IL-6 secretion was drastically enhanced with the presence of trivalent chromium in U937 human monocytes. In addition, the combination (estradiol and chromium) was very effective in inhibiting oxidative stress, monocyte adhesion to endothelial cells, lipid peroxidation and heart disease risks factors (Jain et al, 2004).

II.6 Sulfur-containing compounds

The property of lowering blood sugar has been attributed to onions and garlic (Sheela et al 1992). The active principles are believed to be the sulfur containing compounds, allyl propyl disulphide (APDS) and diallyl disulphide oxide (allicin), although other constituents such as flavonoids may play a role. Experimental and clinical evidence suggests that APDS lowers glucose level by competing with insulin (also a sulfur containing hormone) for insulin-inactivating sites in the liver (Sharma et al 1977). This results in an increase of free insulin. APDS administered in doses of 125 mg/kg to fasting humans caused a marked drop in blood glucose levels and an increase in serum insulin. Allicin at doses of 100 mg/kg produced similar effects (Sharma et al 1977). In addition many hypoglycemic oral drugs are sulfonylureas that are known to lower plasma glucose by stimulating insulin secretion and to a lesser extent, by improving peripheral and hepatic insulin sensitivity.

CHAPTER III

METHODS

III.1 Animal study

KK/HIJ mice experiment

We used an animal model in order to have a better understanding of the mechanisms of action of fonio and/or fonio extracted compounds metabolisms in selected living animals. Animal Model Used: KK/HIJ Mice. As previously noted, this study aimed to investigate diabetes and its complications on an animal model and the the KK/HIJ mice that were first produced by Nakamura and coworkers in 1967 (Kanasaki et al 2011). According to them, the " KK/HIJ mice are a very good model of human diabetes. They develop a polygenic syndrome of moderate obesity, hyperinsulinemia, hyperglycemia, and hyperlipidemia. Like humans with diabetes, they develop renal, retinal, and neurologic complications. They also undergo a pre-diabetic stage characterized by mild to moderate glomerulosclerosis accompanied by proteinurea. Furthermore, they do not require either insulin or other medications for survival, allowing investigators to study how diabetes progresses without the inference of drugs (Igel et al. 1998; Reddi and Camerini-Davalos 1988; Tatlor et al. 1999)." They also mentionned that similarly to the DBA mice, they are more prone to develop nephropathy than are four other inbred strains. We also think that the renal, retinal, and neurologic complications will result in a production of pro-inflammatory cytokines as observed in human diabetes.

Diet Composition for the KK/HIJ. The diets were prepared by Harlan Laboratory. For diets II and III, 30% of fonio and 30% of rice were respectively combined to 70% of AIN93 (for whole composition of the diets, refer to annex 3). Vitamins and mineral contents but chromium were accounted for and adjusted as required for the mice diets.

Mice Methods. A total of sixty K-K/TA mice (3-4 week of age) were used and assigned to one of 3 diets during a two-month trial period:

Group I or control: AIN93 diet (n =20);

Group II: AIN93 +Fonio diet (n =20);

Group III: AIN93 + rice diet (n =20).

Mice were fed with the different diets for 49 days and sacrified to investigate mechanisms involved in principal tissues (heart, liver, spleen, lungs, and kidney) and cytokines assayed following the ELISA method. Mice were anesthetized by CO_2 inhalation for 2-3 minutes until unconscious (not moving but still breathing) and blood was drawn from the retro-orbital plexus for the periodical determination of plasma glucose and insulin, triglycerides (TGs), LDL and HDL by the Clinical Analyzer. Microcultures on liver, lungs and spleen cells were performed for cytokines secretion: mitogens at different doses were added to 1×10^6 viable cells in fetal calf serum medium (FCS) and allowed to incubate in a 5% CO_2 incubator at 37 degrees Celcius for 48 hours, and cytokines were assayed on supernatants using ELISA kits. The whole protocol modified by Dr Kuvibidila (personal information) is described in The whole protocol is described in **Appendix 1**. Prior to sacrifice day, insulin activity was assessed by the oral glucose tolerance test (OGTT). After a fasting period of 18 hours, mice were fed with glucose at dose of 2-g/kg body weight with a gastric gavage at 10:00 a.m. Then 15

microliters of blood were extracted from the caudal vein at 0, 30, 60, 120 min at weeks 4, 6 and 8. Measurements of blood glucose were made immediately using the Clinical Analyzer.

CD1 mice experiment

These normal healthy mice were used to test the immuno-stimulatory effects of fonio extracts. Seven normal healthy male mice, 2 months of age, were provided by the Laboratory Animal Resources (LAR) of Oklahoma State University. Early in the morning mice were randomly assigned in two groups of 3 and 4. After recording their weights, mice were left to accommodate for two hours. After the accommodation period, the treatment group (n=3) received by intraperitoneal injection (IP) 10 mg of the fonio extract in 100 µl PBS solution and the control group (n=4) received an equal amount of PBS solution without fonio extracts by IP injection. Thirty minutes later each group of mice received 5 µg of LPS in 100 µl PBS to mimic the septic shock. Two hours after the LPS injection, mice were sacrificed. Organs and blood were collected. After centrifugation of blood, both plasma and weighed organs were stored at -80ºC. A week later, spleen cell suspensions (2 x 10^6/ml) were activated with 2.5 µg/ml LPS for 48 h. Cytokines in plasma and spleen cell supernatants were measured by enzyme immunosorbant (ELISA).

III.2 Cell culture study

Fonio extract preparation. Extraction of the total beta-glucan from fonio was achieved following the method developed by Carr and his colleagues which consists of a 16-hour extraction with NaOH = 1N at room temperature followed by its neutralization by HCl= 1N. The neutralized mixture with a pH of 7.2 was centrifuged and the supernatant rich in

beta-glucan, minerals and some proteins were freeze-dried into a brown residue. See the diagram of the Carr Method in annex 2.

Ten mg of the residues composed of total beta-glucan and minerals were dissolved in 10 ml of a complete calf serum medium to be used as stock solution. A second stock was further obtained through a 1/10th dilution of the first stock solution; solution 2 was used for concentrations ranging from of 1 to 5 µg of extract, and solution 1 for concentrations in the range of 10 to 100 µg for the treatment of the THP1 cell culture line.

THP1 Cell Proliferation Methods.The effects of fonio extracts on THP1 Cell proliferation and survival was quantified by the colometric method developed by Mosmann (1983) and revised by Kuvibidila (1992,1998,and 1999). The method uses a tetrazolium salt MTT (3-(4,5- dimethylthiazol-2,5-diphenyl tetrazolium bromide, of which the ring can be cleaved within the mitochondria of only living and viable cells leading to a chromophore that can be quantified at a wavelenght of 570 or 575 nm. The whole protocol is described in **Appendix 1**.

CHAPTER IV: ARTICLE I

EFFECTS OF DIGITARIA EXILIS (FONIO) ON GLUCOSE METABOLISM

AND INFLAMMATORY CYTOKINES IN KK/HIJ DIABETIC MALE MICE

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Short running title: *Digitaria exilis* effects on glucose metabolism and inflammation in KK/HIJ diabetic mice

Abstract

Digitaria exilis (fonio), a wild cereal consumed in West Africa, is rich in chromium, β-glucans and sulfur amino acids, factors known to modulate inflammation. Fonio is thought to have anti-diabetic properties; however the mechanisms are unknown. We hypothesize that it might modulate diabetes pathogenesis through improvement of insulin resistance, glucose metabolism, and reduced inflammation. To test our hypothesis, 4-week old KK/HIJ transgenic male mice were fed either the AIN 93 diet or the same diet fortified with 30% fonio or rice flours (n=20/group). Ten mice per dietary treatment were sacrificed after either 3 or 4 months of feeding. Glucose and insulin tolerance tests (GTT & ITT) were studied by standard techniques 7 days prior to euthanasia. After CO_2 inhalation and prior to euthanasia, blood samples were collected from the retro-orbital plexus, centrifuged at 400 x g and serum was collected and immediately frozen at -80ºC. To assess the effects of various dietary treatments on the secretion of inflammatory cytokines, 2×10^6/ml viable spleen cells were activated with 2.5 µg/ml of bacterial lipopolysaccharides (LPS) or plain medium for baseline for 48 h. Supernatants were collected after centrifugation of the cultures at 400 x g, 4ºC, for 10 min, aliquoted and frozen at -80ºC until analyzed. Serum and supernatant inflammatory cytokines [Interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor–alpha (TNF-α)] were assayed by enzyme linked immunosorbant (ELISA) with commercial kits. After 4 months of feeding, results obtained from insulin tolerant test (ITT) were different among dietary treatment groups. Although dietary treatment affected feed intake, neither fonio nor rice significantly affected growth rate. Mean blood glucose levels were lower in mice fed fonio-fortified diet than in those fed the other two diets (p<0.01). Fonio non-significantly decreased mean GTT values. Mean serum TNF-α levels were lower in mice fed fonio-fortified diet than in those fed baseline and/or rice-fortified diets (p<0.05). Mean serum IL-6 levels of mice fed fonio-fortified diet were not different from those fed the baseline diet; but they were lower than those fed rice-fortified diet (P<0.006). Fonio and rice did not significantly affect *in vitro* IL-6, TNF-α and IL-10 secretion; however IL-1β was significantly reduced in the non-activated spleen cells of the fonio group. Data suggest that fonio may modulate diabetes severity by improving insulin tolerance and reducing low grade inflammation.

Key words: fonio, type 2 diabetes, KK mice, cytokines, GTT, ITT, inflammation, IL-1, IL-6, IL-10, TNF-alpha

Introduction

Digitaria exilis, mainly known as fonio and confined to West African diets, is currently among the promising emerging edible crops in the world, not only because of its taste but mainly due to its alleged potential therapeutic properties. The Agriculture and Natural Resources Management Program or Wula Nafaa (WN), a USAID agricultural project is completing a trade agreement between fonio producers and the US government, through its component referred to as GPFo (Fonio Producers' Group). For centuries the cereal was consumed during ceremonial events and empirically used for diabetes care in West African countries. The cereal is reported to be different from other cereals because of its high content of essential sulfur-containing amino acids, specifically methionine and cysteine (Sartelet et al 1996; Delumen et al 1993; Becker et al 1986). Apart from the important contents of sulfur-containing amino acids confirmed by the study we conducted in 2004 (unpublished data), we also found high important amounts of beta-glucans and chromium. All three components are known to exert beneficial physiologic effects on chronic diseases and to boost the immune system through the modulation of inflammatory markers (Volman et al. 2007; Keiss et al, 2003; Chang et al, 2010; Brown and Gordon, 2003; Burkitt et al 1981).

Beta-glucans. Beta-gucan is one of the biologically active compounds found in various cereals including fonio. Previous studies on beta-glucans revealed that the potency of their physiological effects depended on the physico-chemical characteristics of each type. Most studies found in the literature were confined to beta-glucans extracted from oat, barley and mushrooms. To date, we are unaware of any studies investigating the effects of fonio beta-glucans on any physiological parameter.

Depending on the source, there are clear differences in macromolecular structure between β-glucans. The cell wall β-glucans of yeast and fungi consists of 1,3 β-linked glycopyranosyl residues with small numbers of 1,6 β-linked branches. In contrast, cereal cell walls contain unbranched β-glucans with 1,3 and 1,4 β-linked glycopyranosyl residues, whereas β-glucans from bacterial origin are unbranched 1,3 β-linked glycopyranosyl residues (Volman et al. 2007. Furthermore, besides differences in type of linkage and branching, β-glucans can vary in solubility, molecular mass, tertiary structure, degree of branching, polymer charge and conformation (triple or single helix, or random coil). All these characteristics may differently influence several physiological parameters.

As early as 1981, Simpson and his colleagues (1981) showed that these types of fibers were capable of slowing the digestion and absorption of carbohydrates, thereby preventing rapid rises in blood glucose and increasing the sensitivity of tissue cells to insulin and thus preventing an excessive secretion of insulin. Jenkins et al (1980) and Vahouny et al (1982) showed that patients who were supplemented with 14 and 26 grams of a beta-glucan-rich fiber per day, required less insulin and displayed milder glycosuria. In addition, both crude fibers and total dietary fibers were reported to contribute to the lowering of the glycemic index of foods (Jenkins et al, 1980; 1981; 2002) and were inversely related to body mass index (McKeon et al 2002), HbA_{1c} levels and ketoacidosis risk (Toeller 2002). Moreover, fiber consumption reduced urinary C-peptide excretion in healthy people as well as circulating insulin and lipids in hyperlipidemic subjects (Jenkins et al 2002; Giacco et al 2002).

`Brown and Gordon (2003) have recently suggested that high molecular weight β-glucans from fungi directly activate leukocytes, while low molecular weight β-glucans only modulate the response of cells when they are co-stimulated with pro-inflammatory cytokines. With respect to the characteristics of β-glucans, it should also be noted that the isolation method may also influence these properties (Volman et al 2007). Due to these characteristics, they can behave as different biological ligands for cell receptors. When soluble and non-soluble portions of beta-glucans are administered orally, they are taken up by intestinal macrophages for antigen presentation to, and activation of immune cells in the spleen, lymph nodes and bone marrow (Hong et al 2004). Once in the bone marrow, large molecular weight beta-glucans are reduced into lower molecular weight fractions that will bind receptors such as dectin-1 or complement receptor-3 to induce the immune responses including cytokines secretion (Hong et al 2004).

Fiber rich beta-glucan consumption reduced urinary C-peptide excretion in healthy people as well as circulating insulin and serum lipids in hyperlipidemic subjects (Jenkins et al 2002; Giacco et al 2002). In trauma patients, beta-glucans were shown to induce plasma concentrations of IL-1 while they had no effect on those of TNF-alpha (Browder et al 1990). However, in contrast to Browder's study, Lehne et al. (2006) found no difference in cytokine and immunoglobulin concentrations in the blood of trauma patients after 5 days treatment with three different concentrations of yeast β-glucan compared to baseline, although IgA concentrations were increased in saliva.

Chromium. Currently, it is known that chromium is a vital trace element for glucose metabolism. Studies demonstrated that it is a cofactor of insulin that facilitates its action in the uptake of glucose into cells. Without chromium, insulin action is blocked and

glucose levels are elevated (Anderson 1992; Mooradian et al 1994). Supplementing the diet with chromium decreased fasting glucose levels, improved glucose tolerance and lowered insulin levels in humans. In addition, total cholesterol and triglyceride levels decreased while HDL-cholesterol levels increased (Anderson 1992; Mooradian et al 1994; Chen et al, 2009). Also, Vincent and his colleagues made a major breakthrough regarding the structure and role of chromodulin or low-molecular-weight-chromium-binding substance. They showed that the oligopeptide, composed of residues of glycine, cystein, glutamate and aspartate binds 4 atoms of chromium. The resulting holo form can potentiate 7 times the action of insulin without increasing its concentration by binding the insulin receptor-kinase; and interestingly, insulin stimulation was directly dependent on the number of bound chromium atoms (Vincent, 1999; Vincent, 2000). More recently, Chen and coworkers showed that chromium supplementation in milk powder improved diabetes by enhancing insulin signaling in skeletal muscle through a decrease in IRS1-Ser307 (Chen et al 2009).

Jain and Krishnaswamy (2001) demonstrated for the first time that chromium chloride inhibited oxidative stress and TNF-alpha in U-937 monocytes (in vitro); according to the authors, TNF-alpha was associated with decreased insulin sensitivity (in vivo) in diabetes. Furthermore, Jain and his colleagues showed that the reducing effect on inflammation by 17-beta-estradiol through inhibition of IL-6 secretion was drastically enhanced by the presence of trivalent chromium in U937 human monocytes. In addition, the combination (estradiol and chromium) was very effective in inhibiting oxidative stress, monocyte adhesion to endothelial cells, lipid peroxidation and heart disease risks factors (Jain et al, 2004).

Sulfur containing amino acids. Fonio is relatively rich in sulfur-containing amino acids and some sulfur-containing compounds had anti-diabetic properties. For instance, allyl propyl disulphide (APDS) and diallyl disulphide oxide (DADS or allicin), found in garlic and onions had the property of lowering blood glucose (Sharma et al, 1977). Experimental and clinical evidence suggests that APDS lowers glucose level by competing with insulin (a sulfur containing hormone) for insulin-inactivating sites in the liver (Sharma et al, 1977). This results in an increase of free insulin. APDS administered in doses of 125 mg/kg to fasting humans caused a marked drop in blood glucose levels and an increase in serum insulin. Allicin at doses of 100 mg/kg produced similar effects (Sharma et al 1977). In addition many hypoglycemic oral drugs are sulfonylureas that are known to lower plasma glucose by stimulating insulin secretion and to a lesser extent, by improving peripheral and hepatic insulin sensitivity. In a recent study, the oil-soluble DADS extracted from garlic were shown to efficiently remove the endotoxin LPS, reactive oxidants in plasma and to inhibit platelet adhesion (Chang et al, 2010). Also, NF-κB activity in blood samples treated with unfertilized or sulfur-fertilized garlic extracts was reduced by 25%, and 41%, respectively, thus promoting an anti-inflammatory environment by cytokine modulation in the surrounding tissues (Keiss et al, 2006). There is little, if any information on the effects of fonio on in vivo or in vitro secretion of pro-inflammatory cytokines. Inflammation is a common feature of type 2 diabetes and is thought to play a crucial role in the disease pathogenesis, either by altering insulin sensitivity or by induction of apoptosis of beta cells in the pancreas. We have observed that West African patients who regularly consume fonio, experience fewer symptoms such as joint pain or weight gain associated with type 2 diabetes. However, neither we,

nor other investigators that work in the field of glucose metabolism and type 2 diabetes, have ever investigated the mechanism by which fonio modulates these symptoms . In the present study, given the high levels of beta-glucan, chromium, and essential sulfur amino acids and their properties in improving glucose metabolism and immuno-regulation, we hypothesized that fonio would improve glucose and insulin tolerances, and modulate inflammation in diabetic KK/HIJ mice. We defined modulation of inflammation as either a decrease in pro-inflammatory cytokines or an increase in anti-inflammatory cytokines in the presence or absence of a stimulus. The AIN 93 diet, the AIN-supplemented fonio and AIN-supplemented rice diets were used to test their effects on glucose metabolism, insulin sensitivity and selected inflammatory cytokines in a mouse model.

KK/HIJ transgenic mice developed by Nakamura and coworkers in 1967 (Kanasaki et al 2011) were used to test our hypothesis because they develop a polygenic syndrome of moderate obesity, hyperinsulinemia, hyperglycemia, and hyperlipidemia (Nakamura and Yamada 1963; Suto et al 1998; Chen et al 2009), characteristics similar to the human disease. Like human diabetic patients, they develop renal, retinal, and neurologic complications accompanied by an elevated production of pro-inflammatory cytokines. They also undergo a pre-diabetic stage in which they do not require either insulin or other medications for survival, allowing investigators to study how diabetes progresses without the inference of drugs (Igel et al 1998; Reddi et al 1988).

Materials and methods

Samples. Fonio, was obtained in the suburb of Dakar, Senegal, from Maria Distribution and the white rice was purchased from a supermarket in Stillwater, Oklahoma, USA.
Diet Composition for the KK/HIJ. The diets were prepared by Harlan Laboratory. AIN 93 (Diet I) was used as control diet. Diet II was obtained by supplementing AIN 93 with

30% of fonio and Diet III with a supplementation of 30% rice. Apart from chromium, vitamins and mineral contents were accounted for and adjusted as required for the mice diets. Chromium contents were left as they naturally occurred in the diets. **Table 1** displays the composition of the AIN 93 G diet and in **Table 2** and **Table 3,** the mineral mix and vitamins contents of the AIN G are shown, respectively.

TABLE 1

AIN-93G diet formulated for the growth, pregnancy and lactational phases of rodents

Ingredient	
	g/kg diet
Cornstarch	397.486
Casein (≥85% protein)	200.000
Dextrinized cornstarch	
(90–94% tetrasaccharides)[1]	132.000
Sucrose	100.000
Soybean oil (no additives)	70.000
Fiber[2]	50.000
Mineral mix (AIN-93G-MX)	35.000
Vitamin mix (AIN-93-VX)	10.000
L-Cystine	3.000
Choline bitartrate (41.1% choline)[3]	2.500
Tert-butylhydroquinone	0.014

[1]Dyctrose (Dycts, Bethlehem, PA) and Lo-Dex 10 (American Maize, Hammond, IN) meet these specifications. An equivalent product may also be used.
[2]Solka-Floc®, 200 FCC (FS&D, St. Louis, MO) or its equivalent is recommended.
[3]Based on the molecular weight of the free base.

Table 1, Table 2 and 3 were copied from the article by Reeves and colleagues.

TABLE 2

Contribution of mineral elements to the AIN-93G and AIN-93M diets when the recommended mineral mixes AIN-93G-MX and AIN-93M-MX, respectively, are fed at 35 g/kg of the diet

	Diet	
	AIN-93G	AIN-93M
	mg/kg diet	
Essential mineral element		
Calcium	5000.0	5000.0
Phosphorus[1]	1561.0	1992.0
Potassium	3600.0	3600.0
Sulfur	300.0	300.0
Sodium	1019.0	1019.0
Chloride	1571.0	1571.0
Magnesium	507.0	507.0
Iron	35.0	35.0
Zinc	30.0	30.0
Manganese	10.0	10.0
Copper	6.0	6.0
Iodine	0.2	0.2
Molybdenum	0.15	0.15
Selenium	0.15	0.15
Potentially beneficial mineral element		
Silicon	5.0	5.0
Chromium	1.0	1.0
Fluoride	1.0	1.0
Nickel	0.5	0.5
Boron	0.5	0.5
Lithium	0.1	0.1
Vanadium	0.1	0.1

[1] A total of 3000 mg P/kg diet is recommended for each diet. The difference between the contribution of the mix and the recommended dietary amount is made up from the contribution of phosphorus from casein.

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Table 3. Estimated minimal vitamin and other nutrients composition of AIN-93G and 93-M contents.

Estimated minimal nutrient composition of AIN-93G and AIN-93M rodent diets[1]		
Vitamins		
Nicotinic acid, *mg*	30.0	30.0
Ca pantothenate, *mg*	15.0	15.0
Pyridoxine, *mg*	6.0	6.0
Thiamin, *mg*	5.0	5.0
Riboflavin, *mg*	6.0	6.0
Folic acid, *mg*	2.0	2.0
Biotin, *mg*	0.2	0.2
Vitamin B-12, µg	25.0	25.0
Vitamin K, µg	900.0	860.0
Vitamin E, IU	75.0	75.0
Vitamin A, IU	4000.0	4000.0
Vitamin D, IU	1000.0	1000.0
Other nutrients		
Choline, *mg*	1000.0	1000.0

[1]Values are based on estimates of the nutrient composition of individual ingredients in a nonpelleted formulation.

[2]The estimate of caloric content was based on the standard physiological fuel values for protein, fat, and carbohydrate of 4, 9 and 4, respectively.

[3]Includes L-cystine addition to diet.

Fonio analysis. Crude fiber was determined using the Fibertec system E (Tecator) consisting of a Hot Extraction Unit 1010 (in which an acidic digestion followed by an alkaline digestion were performed), a Cold Extraction Unit 1011 and accessories. Total dietary fiber (TDF) was obtained following the enzymatic-gravimetric method by Prosky adopted by Association of the American Chemists (AOAC 1984) in combination with the Fibertec System E (Tecator). Briefly, the principle is based on the use of the thermo-resistant alpha amylase (termamyl), protease, and amyloglucosidase as the active enzymes. Ground samples are digested with the enzymes followed by a precipitation with ethyl alcohol. TDF is calculated as the weight of the precipitates minus the weight of undigested protein and ash. Minerals were determined by Atomic Absorption Spectrophotometry (by flame with deuterium background correction, or by the graphite

furnace AAS with Zeeman background correction) using a AA 5100 (Perkin Elmer,

Massachusetts,

USA. The analysis of the amino acids was performed at Langston University using an

Amino Acid Analyzer (Hitachi L89100 Illinois, USA.

Experimental animals and diets. Sixty 4-week-old male KK/HIJ mice were purchased

from Jackson Laboratory, USA. Animal care was in accordance to protocols established

at Oklahoma State University and approved by the Institutional Animal Care and Use

Committee. Mice were housed in stainless steel wire cages with woodchip bedding, in

temperature- and humidity-controlled rooms on a 12-hour light/dark cycle. After 7 days

of adaptation, mice were randomly separated into 3 dietary treatment groups

(N=20/group): a. the AIN 93 diet; b. the same diet fortified with 30% fonio flour; or c.

the same diet fortified with 30% rice flour. Body weights and feed consumption were

weekly recorded. Fifty percent of mice (n=10) in each dietary treatment group were

sacrificed after 3 months and 50% after 4 months of feeding

GTT determination. The test was performed 7 days before sacrifice. Mice were

transferred into single cages with woodchip bedding and fasted for 8 hours and weighed

an hour before the test. After an overnight fast, a blood sample ($\leq$ 50 µl) was drawn from

the tail vein for baseline blood glucose assessment, using a glucometer (One Touch;

LifeScan, California USA). This was followed by injection of D-glucose (Sigma) into the

peritoneal cavity. Each mouse received 1 µg glucose /g body weight (1 µg/10 µl water).

Tail blood samples were collected at 10, 20, 30, 60, 90 and 120 min after the injection for

blood glucose measurements.

ITT determination. This test was performed two days before sacrifice. Similarly to the GTT, mice were transferred into single cages with woodchip bedding and fasted for four hours. They were weighed an hour before the test. Right after fasting, baseline blood glucose was determined from blood drawn from the tail vein, using a glucometer (One Touch). Then human insulin (humalin) at the dose of 0.75 units/Kg body weight was injected intraperitoneally, and blood glucose levels were obtained at 15, 30, 60 and 90 minutes.

Spleen cell preparation. Spleens were aseptically removed and immediately transferred in pre-weighed sterile culture tubes containing each 1 ml wash medium (RPMI-1640 supplemented with 25mmol L-glutamine/l 10 g bovine serum albumin/l, 50 mg streptomycin/l and 5 X 10^4 units penicillin/l). After weighing the tubes to estimate spleen weights, single cell suspensions were prepared by gently grinding spleens onto a nylon mesh filter inserted on top of a sterile 50 ml beaker (the piston of a 3ml syringe was used to grind the spleens through the nylon mesh into single cells) as described by Kuvibidila et al (1998). The nylon mesh was washed with 10 ml serum-free RPMI-1640 and the collected cells were transferred to a 15ml conical centrifuge tube. Tubes were centrifuged at 1200 rpm, 24ºC for 10 min. The supernatant fractions were decanted and the obtained pellets were re-suspended in ice-cold sterile deionized water (1 ml) to lyse and get rid of erythrocytes. Further, centrifugation of the tubes was performed twice under the same conditions. The pellets were then resuspended in wash medium (2 ml). Total cell count and viability were determined using the trypan blue staining technique, and enumeration was performed using a hemocytometer under a light microscope.

Spleen cell proliferation. Spleen cells proliferation and viability in the presence or

without E. coli lipopolysaccharide (LPS), were quantified by the colometric method

(MTT) developed by Mosmann (1983) and revised by Kuvibidila (unpublished) as

follows: Viable cells, (2×10^6 per 1000 µl) mixed with or without LPS, were transferred

in triplicate to each well of a 96-well plate (Corning, NY USA) and plates were incubated

at 37ºC, 5% CO_2, in a humidified incubator for 68 hours. Fifty microliters of tetrazolium

salt (3-(4,5- dimethylthiazol-2,5-diphenyl tetrazolium bromide; 5 mg/ml) were added to

each well and the plates were further incubated under the same conditions for 4 h. The

reaction was stopped by adding 50 µl of 0.08 N HCl-isopropanol solution. The test is

based on the fact that (3-(4,5- dimethylthiazol-2,5-diphenyl tetrazolium bromide, for

which the ring can be cleaved within the mitochondria only by living and viable cells,

leads to a chromophore that can be quantified at a wavelenght of 570 or 575 nm.

Spleen cells culture for in vitro production of cytokines. Spleen cells were cultured at a

final density of 2×10^6 viable cells/ml in each well of a 24-well Falcon flat-bottom tissue

culture plate (Corning, NY USA) in RPMI-1640 culturing medium (Sigma Chemical

Co.) containing 10% FCS and with or without the presence of LPS (2.5 µg/mL, 72

hours); incubation was allowed to proceed in a CO_2 incubator. Cell suspensions were

centrifuged at 1200 RPM, 24ºC for 10 minutes. Cell free supernatants were stored at

−80°C for the determination of the proinflammatory cytokines IL-6 and TNF-α.

Stimulatory Index (SI). After being challenged by LPS, SIs of spleens will be determined

by calculation. It is obtained by dividing the value of cytokine produced after LPS

challenge by that obtained before the challenge.

Cytokine assays. Cytokine concentrations in the culture supernatants and serum were determined by enzyme linked immunosorbant assay (ELISA) utilizing mouse Quantikine cytokine kits purchased from R&D Systems (Minneapolis, MN USA). The kit protocols were carefully followed.

Statistical Analysis SPSS 18 was used and a two-factor between-groups analysis of variance (MANOVA) was performed to test differences among groups and to find out whether time had an effect. **Results**

The concentration of biologically active compounds in fonio is summarized in Table 4.

Fibers. Fonio was rich in dietary fiber (15.20 %), crude fiber (3.80%) and beta-glucans (1.53%). Physiologically, the beta-glucan is the most potent among the 3 types of fibers.

Sulfur amino acids. The values of methionine (6.8 %) and cysteine (2%) obtained from our analysis were in the range of those reported by the National Academy of Sciences (4.5 and 2.5% for methionine and cysteine respectively). These variations could derive from the fact that there are many species of *Digitaria exilis* and in addition, the type of soil may be another determinant. When we compare our values to other cereals (obtained from the National Academy of Science), the total sulfur amino acid of fonio (8.8% from our analysis) was more than 3 times higher than those of sorghum (2.6%) and more than 5 times when compared to maize and was almost ten times higher when it was compared to pearl millet. (National Academy of Sciences, 2000).

Minerals. Mean concentrations of iron values (8 mg/100 g) found from the National Academy of Sciences Report (2000) did not agree with our results (National Academy of Sciences, 2000). But they did not precise whether their reported values came from dehulled or whole grains.

We found 2.4 mg/100 g for the dehulled grains and almost ten times the value in the whole grain (22.1 mg/100 g). This may have resulted from the different varieties and/or the nature of soils in which the fonio was grown and also the degree of dehulling.

Table 4: Fonio active components

% Fiber	% Sulfur amino acids:	Trace elements, µg/g
Crude Fiber: 3.80	Methionine 6.80	Chromium 0.87
Dietary fiber: 15.12	Cysteine 2.00	Zinc 25.70
Fonio beta-glucan: 1.5		Copper 4.00
Extracts beta-glucan: 60		Iron 220.81

Feed intake and clinical signs. In **table 5**, clinical changes that occurred in KK/HIJ mice during the trial are described. The 4-week old mice had white and normal fur at the start of the trial but several changes occurred along with the treatments. These changes took place in two different stages. During the first stage, corresponding to the first 3 weeks of treatment, all groups looked normal with no visible physiological changes. The second stage was characterized by a change of color and other visible signs that occurred in the control (AIN) and rice groups. Right after the 3rd week of treatment, KK/HIJ mice in the two groups turned yellow, although they conserved normal fur. During the second, third and fourth month, fur (AIN and rice groups) not only developed into an intense yellow, but in addition became spiky with a dirty-like aspect. The fonio group was not subject to those changes suggesting that supplementation with fonio could block the development of the clinical signs observed with the other groups.

Table 5: Clinical signs of KK/HIJ mice during the trial

		Fur			Color	
	AIN93	Fonio	Rice	AIN93	Fonio	Rice
Week 1-2	Normal	Normal	Normal	White	White	White
Month 1	Normal	Normal	Normal	Yellow	White	Yellow
Month 2	Normal	Normal	Normal	Yellow	White	Yellow
Month 3	Bristling	Normal	Bristling	Yellow	White*	Yellow
Month 4	Bristling	Normal	Bristling	Yellow	White	Yellow
Final	Bristling	Bristling	Bristling	Yellow	White	Yellow

In the fonio group, only one mouse (1) developed these characteristics at the 3rd month but recovered at the 4th month. These changes were very important since they also reflected changes seen in feed intake patterns (**Table 5**) and probably other important physiological changes.

Table 6: Changes in weight gain and feed intakes before and after color change.

	Before color change				After color change			
	Control (g)	Fonio (g)	Rice (g)	*p ≤	Control (g)	Fonio (g)	Rice (g)	*p ≤
Feed intake	$2.67 \pm .08^b$	$3.10 \pm .09^a$	$2.82 \pm .12^b$	.001	$2.79^a \pm .16$	$2.52^b \pm .18$	$2.73^a \pm .17$	.001
BW gain	6.88 ± 0.55	6.47 ± 1.16	6.30 ± 2.35	.95	8.95 ± 1.37	6.53 ± 1.03	7.18 ± 2.25	.21

Values are means ± SEM. *p values reported were obtained after performing paired t tests among the three dietary treatment groups. Means followed by different superscript letters are significantly different; a>b>c; p <0.05.

Before the appearance of the yellow color, corresponding to the first stage (first three weeks), mean food intakes of all groups were recorded at eight time points, and Paired-T Test analyses were performed to test for differences between groups. Feed intake of the KK mice in the fonio group was significantly higher than those of the AIN and rice groups (P =.000 and P = .003 respectively).

During the second stage, from about the third week to the end of the trial, when the AIN and rice groups turned into a deep yellow with spiky and dirty-looking fur, feed intakes for all groups were recorded in 19 time points and paired t-tests were run between groups.

The results showed that the feed intake of the fonio group became significantly lower than those of the control and rice groups (P = .001 and P = .003) at 125 days of treatment. As for body weight gain, neither fonio nor rice affected weight gains (P= 0.21,).

Relative organ weights. Results in **Table 7** show that there was no significant difference between groups (P= 0.095), but there was a trend of higher mean relative liver weights (mg/g body weight) of mice fed fonio-fortified diet as compared to the rice group (P= 0.053) at 95 days. However, the fonio treatment reversed that trend at 125 days and relative liver weights from the fonio groups were no longer different from the other groups (P= 0.73). There was no difference between relative heart weights (mg/g body weights) at 95 days (P=.643) or at 125 days (P=0.11), but a trend (Table 3) of lower mean relative heart weights of the fonio-fed group was observed at 125 days when compared to the rice group (P=.08); There was no difference between the fonio and control groups (P=.93).

Table 7: Mean body weights and mean relative organ weights (mg/g body weight).

95 days of feeding				
	Control	Fonio	Rice	*p ≤

N	10	10	10	
Initial Bd Wt (g)	24.99	23.79	24.14	
Final Bd Wt (g)	37.53 ± 1.61	36.95 ± 1.84	36.08 ± 2.98	
Weight gain (g)	12.54 ± 0.69	13.16 ± 0.64	11.94 ± 1.10	0.59
Liver mg/g Bd Wt	60.20 ± 2.84	66.01 ± 3.79	53.17 ± 4.72	0.095
Kidneys mg/g Bd Wt	16.34 ± 1.45	15.91± 1.67	15.60 ± 1.19	0.48
Spleen mg/g Bd Wt	4.41 ± 0.32	3.76 ± 0.21	3.78± 0.28	0.18
Heart, mg/g Bd Wt	5.39 ± .18	5.33 ± .14	5.55 ± .12	0.64
Lungs, mg/g Bd Wt	5.94 ±.77	6.31 ±.73	6.28 ±.66	0.67
125 days				
Initial Bd Wt (g)	23.84	24.44	24.34	
Final Bd Wt (g)	38.50 ± 2.40	34.84 ± 2.69	37. 45 ± 2.86	
Weight gain (g)	15.93 ± 0.50	11.06 ± 0.4	13.47± 0.45	0.11
Liver, mg/g Bd Wt	63.78 ± 3.44	57.77 ±4.01	59.99 ±5.32	0.59
Kidneys, mg/g Bd Wt	14.78 ± 1.67	15.03 ± .68	15.51± 1.29	0.50
Spleen, mg/g Bd Wt	$4.04 ± 0.26^a$	$3.09 ± 0.10^b$	$3.89 ± 0.19^a$	0.007
Heart, mg/g Bd Wt	5.00 ± .0.10	5.03 ± .22	5.47 ± .22	0.11
Lungs, mg/g Bd Wt	6.28 ±1.19	5.85 ±1.18	5.96 ±1.36	0.64

Values are means ± SEM. P* values reported were obtained after performing MANOVA among the three dietary treatment groups. Means followed by different superscript letters are significantly different; a>b>c; p <0.05.

Dietary treatments had no significant effects on relative kidney weights at 95 days or 125 days (P=.482 and P=.497, respectively), but time had an effect on the rice-fed KK groups since relative kidney weights at 95 days for all groups were significantly higher than those of the KK fed 125 days (P=0.038). After 95 days of treatment, there was no significant difference in relative spleen weights between the 3 groups (P= 0.18). However after 125 days of treatment, MANOVA detected significant differences in relative spleen weights among the three treatment groups (P= 0.007). Specifically, mean spleen weights of mice fed fonio-fortified diet were significantly lower than those fed the control or rice-fortified diets (P = 0.008 and P =0.03 respectively). AIN, fonio and rice supplemented diets had no effects on relative lung weights at 95 days or 125 days (P= 0.67 and P= 0.64).

Insulin Tolerance Test (ITT). Results in **Figure 1** show that at 95 days, there was no

significant difference between the 3 groups in mean blood glucose levels after the ITT

was performed (P=.441).

Figure 1: Glucose levels after ITT at 95 days.
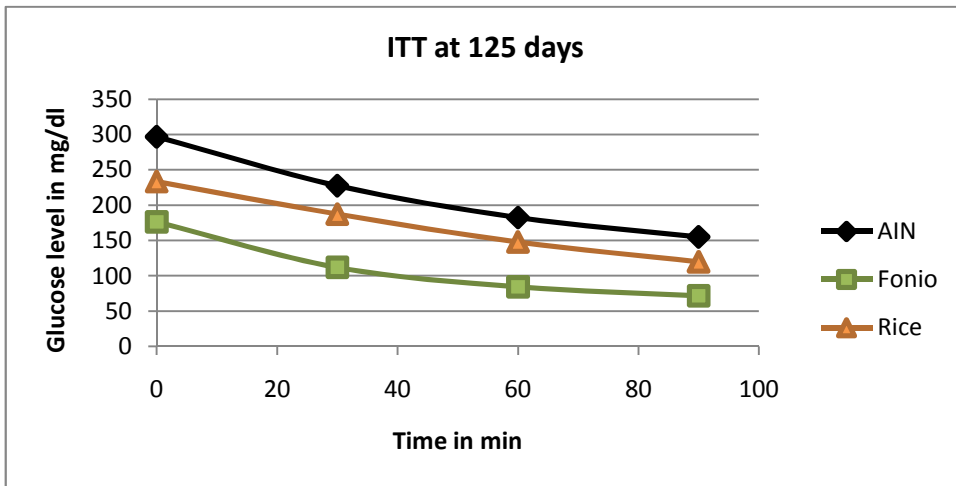
Figure 2: Glucose levels after ITT at 125 days

At 125 days (**Figure 2**), there was a significant difference in glucose levels between

groups after ITT was performed (P =.000) and blood glucose levels of the fonio group

were significantly lower than those of the control and rice groups (P = .000 and P = .008, respectively).

Glucose tolerance test (GTT). After 90 days of treatment (**Figure 3**), none of the three treatments affected glucose clearance after GTT was performed (P= .374).
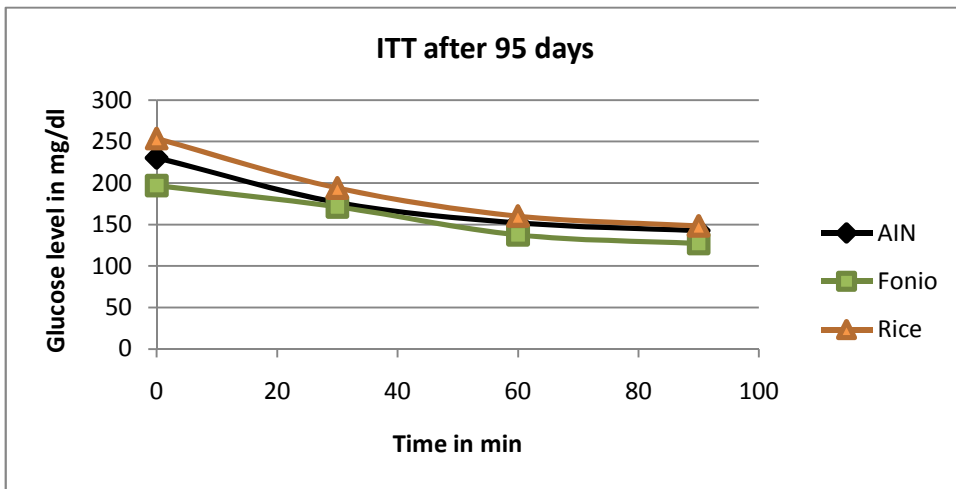

Figure 3: Glucose levels after GTT at 95 days

Figure 4: Glucose levels after GTT at 125 days
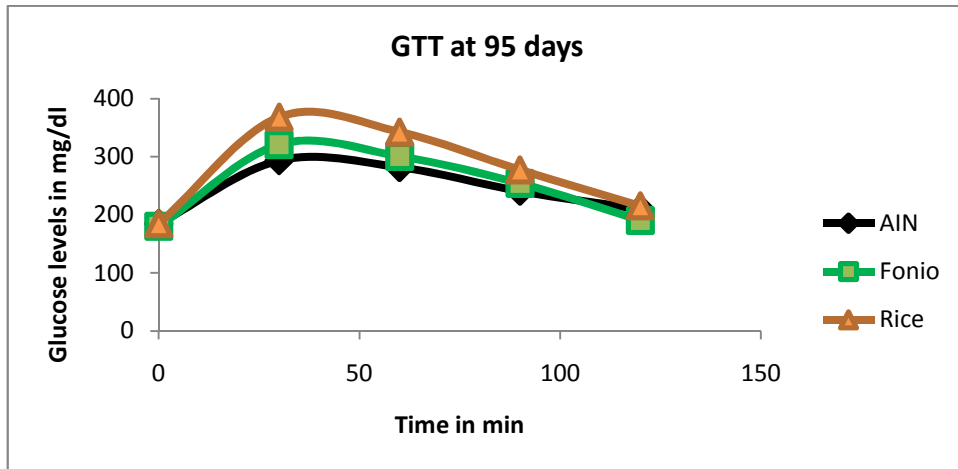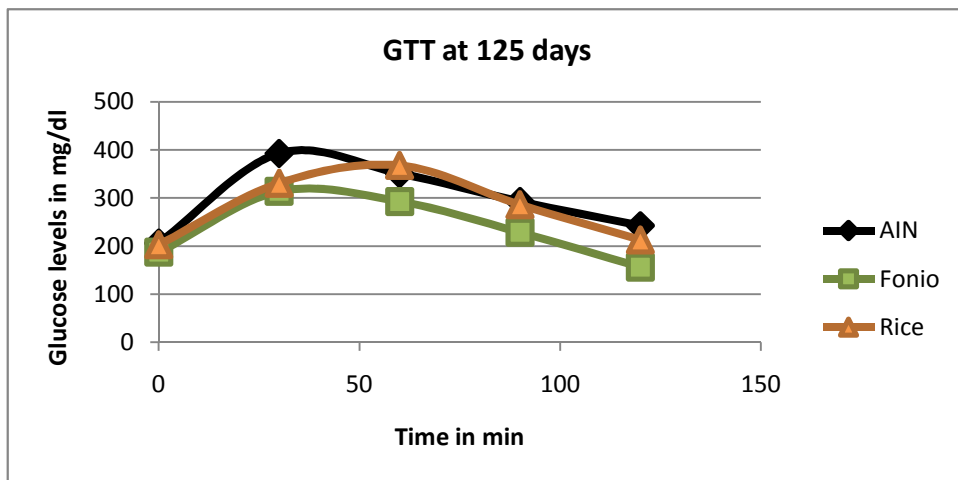
At 125 days **(Figure 4)**, GTT was still not significant but there was a strong trend of

lower mean blood glucose levels of the fonio group as compared to the control and rice

groups (P = 0.057 and P = 0.16, respectively).

In vitro pro-inflammatory cytokines secretion in KK/HIJ mice. MANOVA (2-Way-

between groups) detected significant differences among the three diets at 95 days in

spleen secretion of IL-1 beta (P= .003) without LPS challenge. Also, there was a strong

trend of a lower mean for the fonio group at 125 days of treatment as compared to others

(P=.067). SI of the rice group was lower than the control and fonio groups at 95 days.

There was no difference among SIs at 125 days (**Table 8**).

Table 8: Effect of dietary treatment on IL-1 beta secretion by spleen cells at 95 and 125
days.

	Control	Fonio	Rice	*p ≤
95 days				
No LPS	10.58 ± 2.43^b	10.25 ± 6.33^b	18.33 ± 6.4^a	.003
With LPS	56.63 ± 5.18	54.24 ± 5.70	64.77 ± 5.6	0.78
SI	8.01 ± 2.01^a	6.18 ± 0.7^a	3.76 ± 0.33^b	0.07
125 days				
No LPS	9.77 ± 1.50	7.39 ± 0.71	10.99 ± 1.36	.067
With LPS	76.98 ± 15.1	53.78 ± 8.62	78.75 ± 19.9	.45
SI	8.46 ± 1.63	8.28 ± 1.73	6.90 ± 1.20	.74

Values are means ± SEM. *p values reported were obtained after performing MANOVA among the three
dietary treatment groups. Means followed by different superscript letters are significantly different; a>b>c;
p <0.05.

Table 9: Effect of dietary treatment on IL-6 secretion by spleen cells.

	Control	Fonio	Rice	*p ≤
95 days				
No LPS	30.32 ± 4.49	21.65 ± 11.74	14.03 ± 5.86	.360
With LPS	468.25 ± 47.2	368.75 ± 93.1	451.60 ± 79.3	.709
SI	18.97 ± 3.15[b]	19.06 ± **XX**[b]	32.21 ± 5.79[a]	.017
125 days				
No LPS	36.43 ± 6.10	33.34 ± 6.95[a]	43.70 ± 10.62	.67
With LPS	380.5 ± 28.6[a]	263.13 ± 30.4[b]	475.4± 56.4[a]	.05
SI	16.78 ± 5.40	12.25 ± 4.56	14.94 ± 2.59	.99

Values are means ± SEM. *p values reported were obtained after performing MANOVA among the three dietary treatment groups. Means followed by different superscript letters are significantly different; a>b>c; p <0.05. SIs of the rice is significantly lower than those of the control and fonio groups.

Results in **Table 9** show that IL-6 levels after LPS activation in spleens from fonio fed KK/HIJ mice were significantly lower than those from the control and rice group (P=.05) at 125 days. The SI of the rice group was significantly higher than those of the control and fonio groups (P=.017) at 95 days.

Table 10: Effect of dietary treatment on TNF-alpha secretion by spleen cells

|	Control	Fonio	Rice	*p ≤
95 days				
No LPS (pg/ml)	81.30 ± 4.25	82.64 ± 4.31	87.01 ± 8.14	.95
With LPS (pg/ml)	144.29 ± 13.04	170.90 ± 6.21	191.64 ± 11.61	.23
SI	1.83 ± 0.20	2.09 ± .060	2.3 1 ± .19	.13
125 days				
No LPS (pg/ml)	81.76 ± 13.76	84.87 ± 10.46	109.22± 27.51	.30
With LPS (pg/ml)	196.85 ± 31.94	182.87± 19.57	189.65 ± 27.84	.83
SI	**2.64 ± 0.47**	**2.25 ± 0.23[a]**	**2.24 ± 0.39[a]**	**.69**

Values are means ± SEM. *p values reported were obtained after performing MANOVA among the three dietary treatment groups. Means followed by different superscript letters are significantly different; a>b>c; p <0.05.

The in vitro tests results showed that diets had no effects on TNF-alpha secretion with or without LPS challenge at 95 or 125 days **(Table 10)**

In vivo pro-inflammatory cytokines secretion in KK/HIJ mice. MANOVA (2-Way-between groups) results showed that at 95 days (**Figure 5**), there was no significant difference between treatments

(P = .713). However, at 125 days (**Figure 6**) there a significant difference between treatments (P = .02); specifically, IL-6 levels in sera of fonio-fed KK/HIJ mice were significantly lower than those of rice-fed KK/HIJ mice (P = .006), although there was no difference between the fonio group and control groups

(P = .233). Mice were not challenged with LPS before their sacrifice.
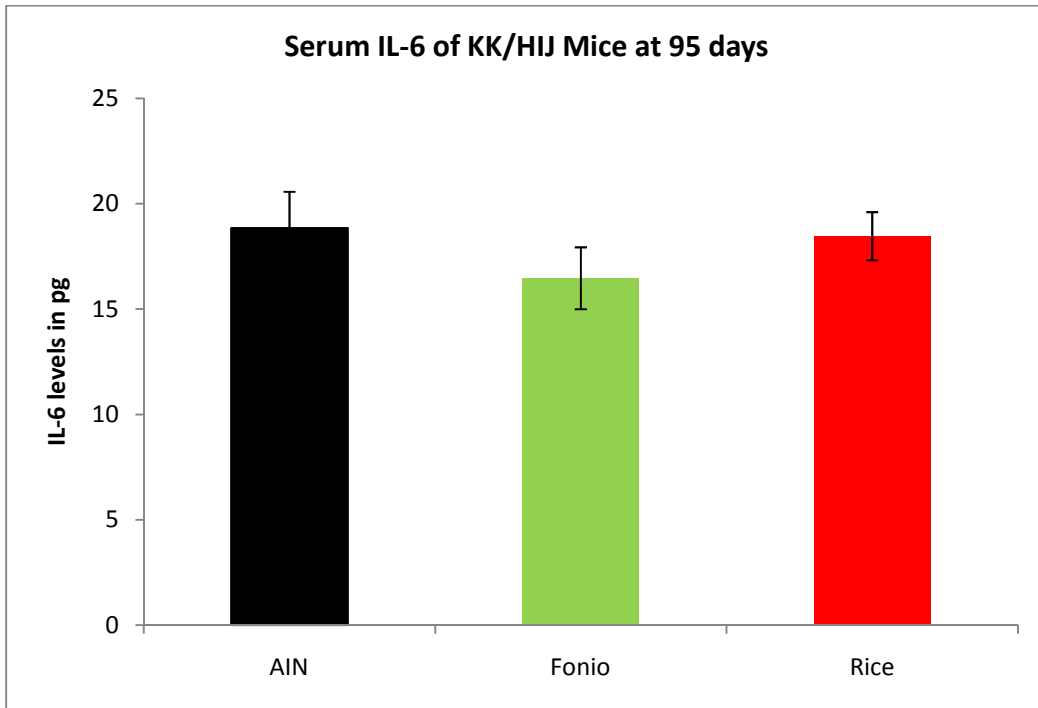
Values are mean ± SEM

Figure 5: Serum IL-6 of KK/HIJ mice at 95 days.
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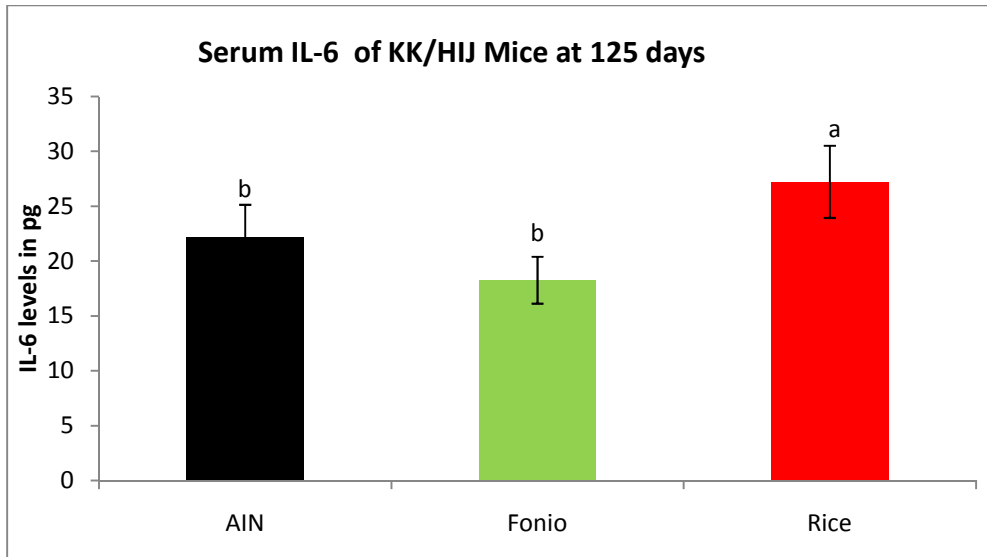
Values are mean ± SEM, n = 10. Bars with different letters are significantly different; a>b; p <0.05.
Figure 6: Serum IL-6 of KK/HIJ mice at 125 days.

Serum TNF-alpha levels. Although there was not a significant difference among groups at 95 days (**Figure 7**), there was a strong trend of lower mean for the fonio group as compared to the control and rice groups (P = .08). However, at 125 days (**Figure 8**), sera TNF-alpha were different among groups (P = .03), specifically TNF- alpha levels of fonio-fed KK/HIJ groups were significantly lower than those of the AIN and the rice-fed groups (P = .01 and P = .05, respectively).
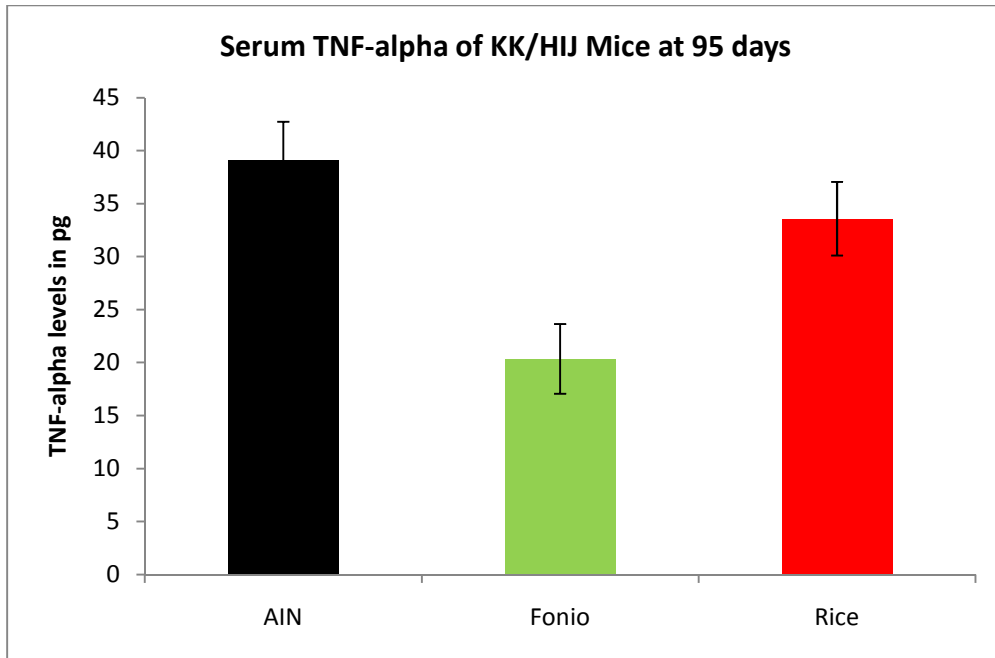
Values are ± SEM, n=10.
Figure 7. Serum TNF-alpha of KK/HIJ mice at 95 days.

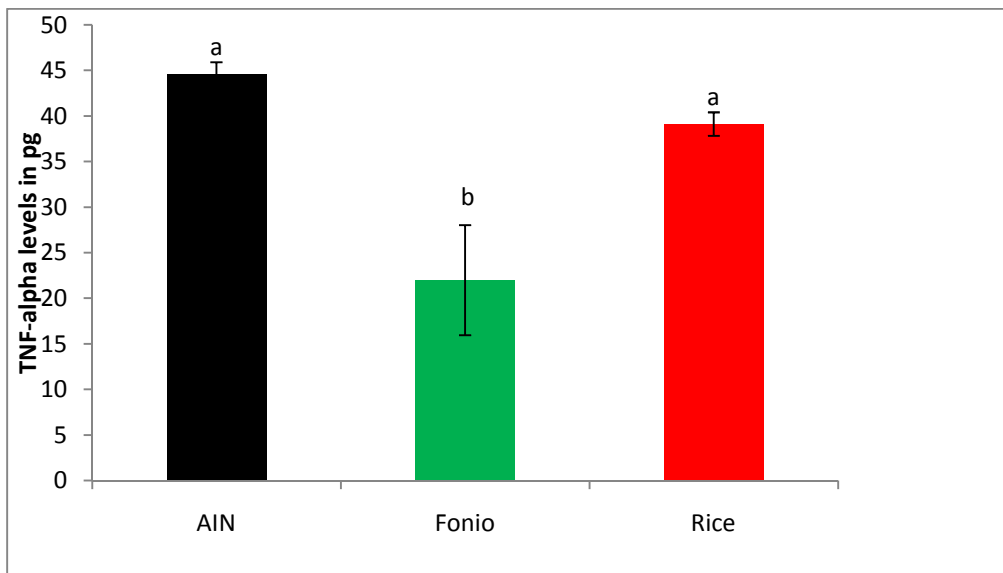Values are mean ± SEM, n = 10. Bars with different letters are significantly different; a>b; p <0.05.
Figure 8. Serum TNF-alpha of KK/HIJ mice at 125 days.

Spleen cells proliferation. MTT was performed on the spleen cells without and with LPS challenge and the percent increase in optical density (OD) was calculated between baselines and treated cells as shown in Table **11**. Spleen cells of the KK/HIJ diabetic mice that were fed fonio-fortified diets for 125 days (**Figure 9**) proliferated much more than those obtained from mice fed the control and rice-fortified diets (P = 0.04).There was no significant difference in percent increase at 95 days, however a strong trend of higher mean percent of fonio-fed group was observed (P = 0.053).

Table 11: Spleen cells proliferation by the MTT test.

	Control	Fonio	Rice	*p
95 days	13.63 ±8.81	16.28 ± 6.50	15.42 ± 8.23	0.053
125 days	12.71 ± 5.20^{b}	45.19± 11.42^{a}	11.58 ± 3.94^{b}	0.04

Means followed by different superscript letters are significantly different; a>b>c; *p <0.

And interestingly, their activity was better controlled after being challenged with LPS. With a higher proliferation rate, they moderately produced quantities of proinflammatory cytokines (IL-6 and TNF-alpha), whereas the rice and fonio groups with a lower proliferation rate, produced too much cytokines in a non-controlled fashion after the challenge insomuch as there was no significant difference in the stimulatory indexes among the three groups.
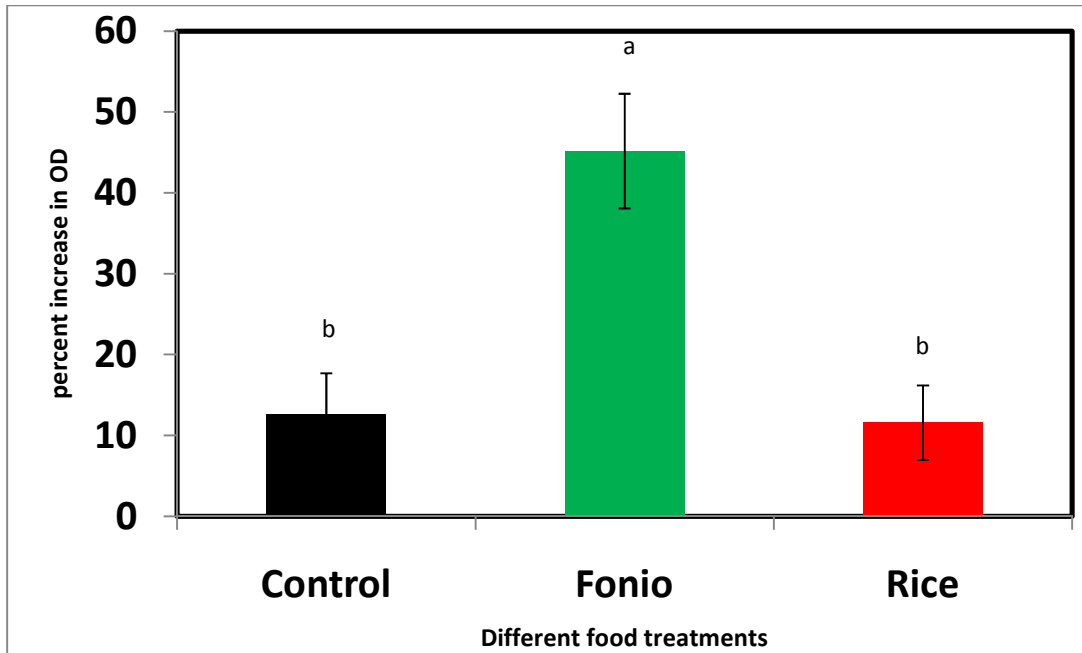
Bars with different letters are significantly different; a>b>c; p <0.05.

Figure 9: Spleen cells percent increase in proliferation before and after LPS challenge.

Discussion

The aim of the present study was to investigate the effects of fonio and rice on diabetes pathogenesis in KK/HIJ mice. A first group of ten mice from each group was sacrificed at 3 months and the remaining at 4 months of treatment. Overall, results indicated that fonio reduced several aspects of diabetes and obesity-related complications. The fonio-fed group displayed different eating patterns, as well as different GTT, ITT and cytokine profiles. All of these factors do contribute to the pathogenesis of diabetes. Important pathological changes were observed with time accompanied by physiological changes that occurred in the control and rice groups and which did not affect the fonio group. Those changes occurred at two specific stages during the treatment period. In the first 3 weeks of feeding, all 3 groups looked normal without any color change or other signs except rapid weight gain which is a specific feature that is inherent to their genetically

modified nature of being bound to growing into obese mice. However, it was interesting

to note that in this stage, feed intakes of the baseline diet-fed (control) and rice-fed

groups were significantly lower than the intakes of the fonio group feed (P = 0.000 and

0.003, respectively)

The second stage, after the first 3 weeks to the end of the trial, was characterized by the

reversed trend in the eating patterns; the rice group was eating more than the fonio group

(P = 0.001) and the AIN group with a higher mean feed intake was eating at least as

much as much the fonio group did (P = 0.003). This sudden hyperphagia, noted in the

rice and control groups, may be due to the fact that the KK/HIJ mice are obtained by

transferring the obese agouti allele to the inbred KK mice and the latter are bound to

develop hyperphagia caused by the development of a strong leptin resistance when they

become obese (Iwatsuka et al 1970). The same eating pattern was observed in humans in

a study in which obese subjects were shown to eat more than the normal (Guss et al

2002)

In addition, this inversed trend in the eating pattern was accompanied by a change in

coloration of the mice; the fur of the hyperphagic groups (rice and AIN) gradually turned

into a faint yellow color characteristic of the KK/HIJ mice when obesity is present. In

fact, the agouti gene encodes a 131-amino acid protein containing a signal sequence

which is produced in the hair follicule playing the role of a strong antagonist to the

melanocyte stimulating hormone (MSH) receptors located on melanocytes. Thus the

hindrance to the melanocytes receptors would inhibit the production of the α-MSH

eumelanin that is responsible for the yellow coloration of the fur (Breyer et al 2005). The

observed changes cited above were exacerbated in the control and rice groups at the end of the trial.

Analysis of the food intake in the KK/HIJ mice showed that nutrition therapy using fonio cereal may at least temporarily block the expression of the obese agouti gene in KK mice through a significant decrease in food intake, not accompanied by a significant weight loss. Only one mouse from the fonio group expressed the yellow color but recovered the following month. That mouse showed signs of sickness throughout the trial. The food intake at the 4th month of the control and rice groups drastically increased corresponding to the strongest yellow coloration of their furs.

Cytokines are known to be physiologically beneficial in trace amounts and can be detrimental to the host if they are over or under expressed in certain cases (Munoz et al 1995). Particularly in diabetes mellitus, presence of low-grade pro-inflammatory cytokines may lead to the aggravation of the disease and later to death if the condition is uncontrolled (Grimble 2005). In that regard, supplementing the diet with fonio peripherally reduced serum-circulating pro-inflammatory cytokines (TNF-alpha and IL-6). After 125 days of treatment, serum IL-6 in the fonio-fed KK group was lower than the rice group (P = 0.006) but was not statistically different from the control (AIN) despite displaying a lower mean. Similarly, at 125 days, serum TNF-alpha levels in the fonio-fed group were significantly reduced when compared to the control (P =0.01) and rice (P =0.05) groups. Because high blood levels of TNF-alpha have been shown to induce insulin resistance in diabetes (Jain and Krishnaswamy 2001), we speculated that the lower concentration of TNF-alpha in mice fed fonio-fortified diet, may at least explain in part the observed improved insulin resistance as shown by the ITT results. After the 125-

day feeding period, when ITT was performed, the areas under the curves for mice fed fonio-fortified diet were significantly lower than those fed the control and rice-fortified diets (P = 0.000 and P = .008, respectively). The presence of high levels of chromium in fonio may further explain the decrease in TNF- alpha production since the same authors (Jain and Krishnaswamy, 2001) demonstrated for the first time that chromium chloride inhibited oxidative stress and TNF-alpha in human U-937 monocytes and that the reducing effect of inflammation by 17 beta-estradiol through inhibition of IL-6 secretion was drastically enhanced by the presence of trivalent chromium in U937 human monocytes.

Thus, in the present study, the mechanisms of action may be attributed to the presence of the above cited active compounds in the cereal; beta-glucans, chromium for its stimulatory effects on the immune system and to a lesser extent the sulfur amino acids that contribute to glucose and insulin metabolism. (Sheela et al 1992). Certain sulfur containing compounds such as allyl propyl disulphide (APDS) and diallyl disulphide oxide (allicin) lower glucose levels by competing with insulin (a sulfur containing hormone) for insulin-inactivating sites in the liver (Sharma et al 1977), leading to a lesser loss of endogenous insulin, resulting in a lower production of the hormone by the body. Although mean spleen weights of mice fed fonio-fortified diet were significantly lower than those of the mice fed the control diet (P = 0.02) and tended to be lower than those from mice fed the rice-fortified diet , MTT results showed that spleens from the fonio group proliferated more than the control and rice groups after LPS challenge. The higher mean weights of spleens from mice fed the control and rice-fortified diets may suggest an early activation due to the presence of elevated circulating serum pro-inflammatory

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cytokines. This phenomenon is usually observed in diabetic and obese mice. Santos and

his colleagues showed that spleen weights in gnotobiotic mice increased after infection

was induced (Santos et al 2007). At baseline (without LPS stimulation), spleen cells of

the fonio group were less activated and expressed less TNF-alpha, IL-6 and IL-1 beta

confirming the trend observed in the sera. High circulating levels of IL-1 beta that are

common in diabetic subjects were significantly reduced *in vitro* in the fonio group at 90

days (P= 0.003) and an excellent downward trend was observed at 125 days (P= 0.067).

It is probable that the presence of high serum concentrations of pro-inflammatory

cytokines at baseline in the control and rice groups may have already activated their

spleens in vivo. Not only are they active blood filters in helping get rid of certain foreign

bodies from the peripheral circulation, spleens also play an active role against

inflammation and infection. This activation generally leads to a higher recruitment and

priming of more splenocytes that may have resulted in heavier spleens in the control and

rice groups. As a result they presented more splenocytes in vivo but the latter were more

exhausted than those from the fonio group that proliferated better when challenged in

vitro because of being less primed by a lower quantity of peripheral low-grade

inflammatory cytokines. Interestingly, spleens cells of the fonio group that proliferated

more, secreted moderate amounts of IL-6 and TNF-α and IL-1 beta after LPS challenge.

Because of their important rate of proliferation, stimulatory indexes from the fonio group

were expected to yield higher SIs, but that was not the case since there was no difference

in SIs among groups suggesting that spleens cells of the rice and fonio groups had to

express more cytokines per unit cell in order to match SIs from the fonio group.

According to Grimble (2008), in certain conditions such as infection and chronic

inflammatory diseases, the host is activated by producing compounds such as cytokines or other chemicals such as nitric oxide and acute proteins. And when the condition is resolved, the body shifts to an inactivation mode which is followed by the clearance of these cytokines or toxic compounds. But a problem with chronic diseases such as diabetes, is that the body fails to fully remove those circulating cytokines. As a result, the body is left with high levels of low grade cytokines that can be detrimental, leading to death in some cases (Grimble, 2008). It is not clear but it looks like that under inflammatory conditions, immune cells of such chronic disease patients overreact by secreting too much cytokines in an uncontrolled manner which makes it difficult for their bodies to completely clear those cytokines even when the condition abates.

Conclusion

In summary, the following are the new findings of the current study:

a. After 4 months of dietary treatment, results showed that supplementation of the diet with 30% fonio can improve insulin resistance and glucose tolerance.

b. 30% of fonio supplementation reduced food intake in KK/HIJ diabetic mice.

c. Supplementing the KK/HIJ mice with 30% fonio blocked the development of their yellow color characteristic of obesity. Therefore, we can infer that fonio may play a role against obesity by blocking the expression of an obese gene.

d. Fonio tended to decrease plasma levels of IL-6, but not TNF-α. Decreased IL-6 will modulate blood levels of C-reactive protein, a marker of inflammation and risk factor for complications of chronic diseases including those of type-2 diabetes.

e. Compared to rice, fonio also tended to decrease in vitro secretion of IL-1β and IL-6, but not TNF-α by LPS activated spleen cells. IL-1β is known to induce apoptosis in cells of islets of Langerhans.

f. Limitations of our study included:

a. Mice were received by groups of ten over a period of 2 months leading us to slightly modify our design.

b. Although we had 3 groups of 20/diet, we further subdivided them in two sub-groups to test the effects of short versus longer exposure to the diets. Therefore some tests (like GTT) lacked adequate statistical power.

c. Important tests such as quantification of the obese gene after treatment would be important but funding was not available for these tests.

In conclusion data suggest that fonio may modulate severity of diabetes by reducing inflammatory cytokines and increasing insulin sensitivity. And very importantly, fonio may play an important role against the onset of obesity.

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CHAPTER V: ARTICLE II

FONIO EXTRACTS CAN MODULATE IN VITRO INFLAMMATORY RESPONSE IN NORMAL CD1 MICE AND THP1 CELLS

Abstract

Background: We recently investigated the effects of fonio-supplemented diet on the pathogenesis of diabetes and inflammation in diabetic KK/HIJ mice. Fonio-supplemented diet was found to modulate inflammatory cytokines and improved diabetes parameters in the mice. The goal of the present study was twofold: a) to investigate the immunostimulatory effects of fonio extracts in non-diabetic healthy CDI mice; and b) tp determine the mechanisms by which fonio extracts modulate cytokine secretion in monocytic THP1 cell line.

Methods: (1) Non-diabetic healthy CD1 mice received an injection in the peritoneal cavity of bacterial lipopolysaccharides (LPS) followed by fonio extracts (mostly composed of beta-glucan) and then were sacrificed 2 h later. Spleen cell suspensions (2×10^6/ml) were activated with 2.5 µg/ml LPS for 48 h.

(2) Monocytic THP1 cells were incubated without and with different concentrations of fonio extracts for 24-72 h. Cell proliferation, viability and differentiation were assessed by trypan blue exclusion, nitroblue tetrazolium salt reduction methods, and microscopy, respectively. Cytokines in plasma and spleen cell and THP1 supernatants were measured by enzyme immunosorbant (ELISA).

Findings and Conclusions: (1) Fonio extracts significantly up-regulated the secretion of TNF-α (pro-inflammatory cytokine) and IL-6 secretion (immune-modulatory – pro- and anti-inflammatory cytokine) *in vivo* (plasma) and *in vitro* (supernatant) in LPS-treated CD1 mice.

(2) Fonio extracts reduced monocytic THP1 cell growth via differentiation and not necrosis. This is the first study showing that fonio can produce immunostimulatory effects in non-diabetic mice and induce cell differentiation of a cell line. The current data allow us to conduct a translational human study.

Key words: CD1 mice, THP1 cell line, inflammation, pro and inflammatory cytokines, cell proliferation, cell differentiation.

1. Introduction

Digitaria exilis, mainly known as fonio and confined to West Africa, is currently among the promising emerging edible crops in the world, not only because of its taste but also due to its alleged potential therapeutic properties. The cereal that has been used for diabetes care in West African countries for centuries (personal communication) is

reported to be different from other cereals because of its higher contents in essential amino acids such as methionine, cysteine (sulfur-containing amino acids) leucine, and lysine. Its amino acid profile, compared to that of whole-egg protein, showed that except for the low score for lysine 46%, the other scores were high: 72% for isoleucine; almost 100% for valine, tryptophan, threonine and phenylalanine; 127% for leucine; 175% for total sulfur and 189% for methionine (Delumen et al 1993; Delumen et al 1986). In addition, our study in 2004 (**Table 1**) showed that fonio contained substantial amounts of beta-glucan, chromium, iron and zinc: compounds known to exert beneficial physiologic effects on chronic diseases (Traore et al 2004) and to boost the immune system through the modulation of inflammatory markers (Volman et al 2007; Brown and Gordon 2003; Burkitt et al 1981). Previous studies on beta-glucans from oat, barley and mushrooms showed significant differences in their physiological effects, depending on their intrinsic characteristics (Brown and Gordon 2003; Hong et al 2004; Jenkins et al 2002). Up-to-date, there is a paucity of data on the effects of fonio extracts (rich in beta-glucan) on inflammatory cytokines.

Depending on the source, there are clear differences in macromolecular structure between β-glucans due to the type of linkages, branching, molecular mass, tertiary structure and isolation methods (Volman et al 2007). These characteristics may influence their immune modulating effects. For example, Brown and Gordon (2003) have recently suggested that high molecular weight (MW) and/or particulate β-glucans from fungi directly activate leukocytes, while low MW β-glucans from fungi only modulate the response of cells when they are stimulated with cytokines. Due to these differences they behave as different biological ligands for cell receptor proteins. Currently, research has

demonstrated that both soluble and non-soluble portions of beta-glucans, orally administered can be taken up by the intestinal macrophages by phagocytosis to be conveyed to the spleen, lymph nodes and bone marrow. Once in the bone marrow, large molecular weight beta-glucans are reduced into lower molecular weight fractions that will bind to dectin-1 receptors or complement receptor-3 to modulate inflammatory cytokines and/or other immune responses (Hong et al 2004).

It is also known that people with diabetes experience a pro-inflammatory cytokines burden (referred to as low grade cytokines) that exposes them to a higher morbidity and mortality when compared to non-diabetic subjects (Tuttle et al 2004). Danzer (2001) reported that peripherally released cytokines (IL-1 and TNF-α) were not only responsible for local inflammatory responses, but also, they synchronize the physiological and behavioral components of the systemic acute phase response to infection.

Our recent in vivo study (manuscript under-preparation) that investigated the effects of fonio on diabetic KK/HIJ mice in a 3 or 4 month period showed that the wild cereal was capable of reducing low grade inflammatory cytokines that are known to be high in obesity and diabetes and which can be detrimental to the host. But the positive results obtained in that study were mainly observed in the 4-month treated mice. The 3-month treatment was not as effective as the 4-month, probably due to a lower exposure to the treatment. In this present study, we wanted to know whether fonio could help during infection/inflammation for many studies showed that infection was a problem among people with diabetes (Davies et al 2005; Cardoso and Salles 2007; Shakov et al 2011) Therefore we decided to run a pilot study whose objective was to treat LPS-challenged CD1 mice with concentrated extracts of fonio to investigate whether:

The CD1 mice treated with the extracts would positively react against the induced inflammation by producing enough pro-inflammatory cytokines to defend themselves. We hypothesized that the fonio extracts treated mice would respond positively; therefore we designed another experiment to investigate:

- The mechanisms by which fonio reduces the secretion of the inflammatory cytokines when inflammation is not present

- The mechanisms by which fonio increases the inflammatory cytokines if inflammation is induced.

For that purpose, an in vitro design is the ideal protocol since it allows assessment of outcomes without the confounders brought about by the various physiological events taking place in vivo.

THP1 cells were used for that purpose. This monocytic cancer cell line was challenged with LPS (simulation of inflammation) and treated with the fonio extract to test its capacity of modulating inflammation by reducing the proliferation rate of cells and cytokine release. Also the mechanisms involved in the process were investigated. THP1 cells are a suitable model for the study because after challenge, they can produce several types of cytokines such as IL-1, IL-6, IL-8 and TNF-α (Samantha et al, 2006), Haversen et al, 2001).

Three objectives were set for the study:

a. THP1 cells with or without fonio were treated with the extracts in the presence of LPS and the 3-(4,5- dimethylthiazol-2,5-diphenyl tetrazolium bromide method (MTT) was run to test the effects of the extracts on the viability and proliferation rate;

b. the Dye Exclusion method was performed on the suspended cells to determine if proliferation rate was decreased by apoptosis or necrosis and;

c. Nitroblue tetrazolium test (NBT) was performed to determine whether or not the decreased growth rate was due to cell cycle arrest induced by differentiation.

If fonio extracts could modulate the production of the above cited cytokines in both CD1 mice and THP1 cells, then we would probably have a first evidence that fonio might help in diabetes care by inducing immuno-stimulatory effects and/or attenuating inflammatory markers.

2. Materials and Methods

3.1 THP1 cell culture line and CD1 mice.THP1 cell line was obtained from the American Type Culture Collection, Rockville, MD, USA and CD1 mice were provided by the Laboratory Animal Resources (LAR) of Oklahoma State University.

2.2 Fonio cereal. Fonio was purchased at Maria Production, one of the biggest suppliers in Dakar, Senegal.

2.3 Chemicals. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma Chemicals (St Louis, MO).

2.4 Fonio extracts preparation and β-glucan determination. Extraction of total beta-glucans from fonio was achieved following a modified method developed by Carr and colleagues (Carr et al 1990) consisting of a 16-hour extraction of of whole fonio flour with NaOH (2g fonio/1ml NaOH) at room temperature followed by its neutralization in HCl. The neutralized mixture with a pH of 7.2 was centrifuged (1000 rpm) and the supernatant rich in beta-glucan s was freeze-dried into a gray residue (**figure 1**). Beta-glucan concentration in fonio extract was determined using Megazyme kits (Megazyme

International Ireland Ltd, Bray Business Park, Bray, Co Wicklow, Ireland) and a 1µg/µl

solution from fonio extract was prepared and kept at -20 ℃ for further use.

Table 1: Fonio active components

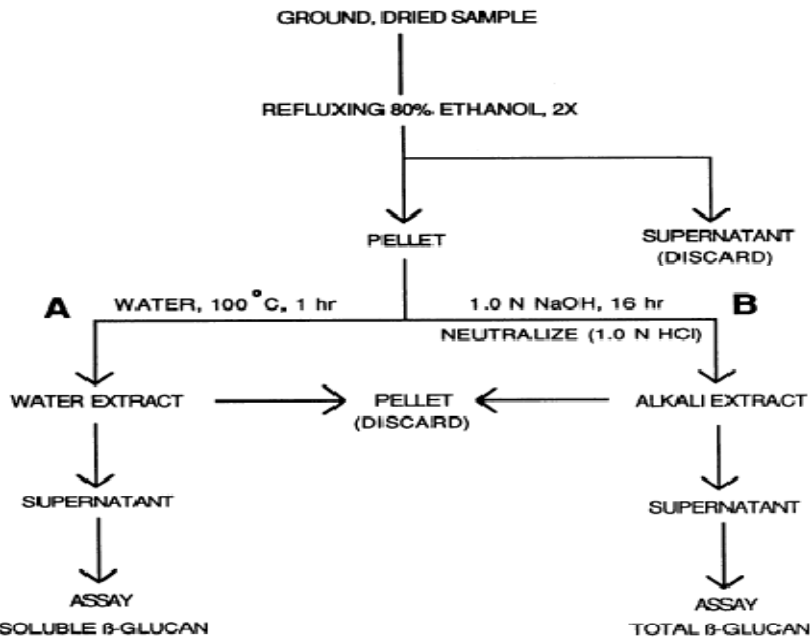| % Fiber	% Sulfur amino acids:	Trace elements, µg/g
Crude Fiber: 3.80	Methionine 6.80	Chromium 0.87
Dietary fiber: 15.12	Cysteine 2.00	Zinc 25.70
Fonio beta-glucan: 1.5		Copper 4.00
Extracts beta-glucan: 60		Iron 220.81

Fig. 1. Quantitative extraction procedure for soluble (A) and total (B)
β-glucan determination in cereal-based food products.

Figure 1: Beta-glucan extraction (This diagram is from Carr et al. 1990).

2.5 CD1 mice. Seven normal healthy male mice, 2 months of age, were provided by

LAR. Early in the morning mice were randomly assigned in two groups of 3 and 4. After

recording their weights, mice were left to accommodate for two hours. After the

accommodation period, the treatment group (n=3) received by intraperitoneal injection (IP) 10 mg of the fonio extract in PBS solution and the control group (n=4) received an equal amount of PBS solution without fonio extracts by IP injection. Thirty minutes later the mice received 5 µg of LPS to mimic the septic shock. Two hours after the LPS injection, mice were sacrificed and organs and blood were collected. Blood was centrifuged, then both serum and weighed organs were stored at -80ºC until analyzed.

2.6 CD1 spleen cells. Spleen cells preparation and proliferation were performed following the method by Mossman (1983) and slightly modified by Kuvibidila et al (1992; 1998; 1999).

2.7 Cytokine assays on serum and spleen supernants of CD1 mice. Cytokine concentrations in the culture supernatants and serum were determined by enzyme linked immunosorbant assay (ELISA) utilizing mouse Quantikine cytokine kits purchased from R&D Systems (Minneapolis, MN USA). The kit protocols were carefully followed.

3.8 THP1 Cell growth and count. Prior to the microculture (MTT) and macroculture (mitogen-fonio-dose response) tests, THP1 cells were allowed to grow in 10% fetal bovine serum under an atmosphere of 5% carbon dioxide at 37 ºC for a for a period of 7 to 8 days; the medium was changed every 72 h in order to grow enough cells to use for the tests.

 The Dye Exclusion method was performed on the suspended cells to determine the viability of THP1 cells that were treated with fonio extracts. For that purpose, 10 µL of the obtained cell suspension and 90 µL of 0.4% trypan blue were transfered into a 1ml Eppendorf tube, and injected into the well of a haemocytometer. Living cells (clear) that excluded the Trypan blue were counted against dead cells (blue) that didn't. Viability of

cells was calculated as a percentage of the living cells over the total count of cells (alive + dead). The viability of cells used for the microculture and macroculture tests was between 85 and 95%.

2.9 Effect of fonio-dose response. Following the method developed by Mossman and slightly modified by Kuvibidila et al (1992; 1998; 1998) colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide test (MTT) was performed on the suspended THP1 cells. An aliquot containing 10^6 THP1 cells was transferred into each of six 15-ml tubes. Then 0, 2.5, 5, 10, 50 and 100 µg/ml of fonio extracts dissolved in fetal bovine serum were respectively added in the six tubes and completed to 1ml with 10% fetal bovine serum. From each tube, 3 replicates of 200 µl were transferred into the wells of a 96-well plate. The plate was incubated under an atmosphere of 5% carbon dioxide at 37 ºC for 68 hours, and 50 µl MTT solution was added in each wells and the incubation period was allowed to proceed for an additional 4 hrs for a total of 72 hours. MTT reaction was stopped by adding 50 µL isopropanol-HCL solution to each well. The optical density (OD) was read at 570 nm with a reference wavelength of 650 nm. Mean ODs of triplicates were obtained to calculate the ratio of OD over the baseline (culture without fonio extracts).

2.10 Effects of fonio on IL-8 and IL-6 secretion by THP1 cells (macroculture). Prior to running the macroculture, several essays (data not shown) were made to find out an adequate concentration of LPS that would induce the secretion of subsequent amounts of cytokines by THP1 cells. A concentration of 2.5 µg per ml of culture containing $2x10^6$ cells yielded very good results.

For the IL-8 experiment, 2×10^6 of viable cells were transferred to each well of three 24-well plates, following a chart including all six levels of treatment: HP1 cell alone; THP1 + 2.5 µg LPS; THP1+ 2.5 µg fonio; THP1+ 5 µg fonio; THP1+ 2.5 µg fonio + 2.5 µg LPS; THP1 + 5 µg fonio+ 2.5 µg LPS. For the IL-6 experiment, concentrations of 1, 2.5, 5, and 10 µg fonio were used. Each well was brought to a volume of 1 ml with 10% fetal bovine serum. Then, plates were placed in a CO_2 incubator set at 37 ºC, 5% CO_2 in a humidified atmosphere. The concentration

After 24 hours of incubation, the first plate was centrifuged at 1000 rpm, 4 ºC, for 10 minutes and supernatants (around 500 µl) from each well were collected in 1.5-ml Eppendorf vials and stored at -80 ºC until cytokines essays. The same procedure was repeated for the second and third plates at 48 hours and 72 hours of incubation, respectively. Cytokines were further assayed by ELISA method.

2.11Combined Dye Exclusion and NBT tests. We wanted to know the mechanisms by which fonio extracts were slowing the proliferation of THP1 cells evidenced by the MTT test. For that purpose, THP1 cells allow to grow in Roswell Park Memorial Institute medium (RPMI) medium for 5 days at 37ºC. Then the viability of the cells was determined with the dye exclusion method as described in 2.6. THP1 cells with viability between 85 and 95% were incubated with fonio at different concentrations for in a 24-well plate. After 72 hours of incubation cells were recounted to determine their new viability. If the viability before and after treatment were the same, it would be concluded that the extracts did not induce apoptosis. Also, NBT test would be performed to investigate whether fonio was inducing cell differentiation to slowing down cell proliferation.

Briefly to run the NBT test, THP1 cells were incubated for 5 days with or without fonio. On the 5th day Cells were pelleted and re-suspended in a RPMI-1640 solution of NBT and incubated for an hour at 37ºC. Then cell were re-pelleted and dissolved in Dimethyl sulfoxide (DMSO). Optical densities were read at 540 nm.

Table 2: NBT chart for THP1 cells treated with different fonio extracts.

	1	2	3	4	5	6
A	0 µg Fonio +THP1	0 µg Fonio +THP1 + Vit D	0 µg Fonio +THP1 + RA	0 µg Fonio +THP1 + PMA	Vit D + RA +THP1	Vit D + RA + THP1 + 2.5µg Fonio
B	2.5µg Fonio +THP1	2.5µg Fonio +THP1 + Vit D	2.5µg Fonio +THP1 + RA	2.5µg Fonio +THP1 + PMA	Vit D +PMA +THP1	Vit D + PMA +THP1+ 2.5µg Fonio
C	5 µg Fonio +THP1	5 µg Fonio +THP1 + Vit D	5 µg Fonio +THP1 + RA	5 µg Fonio +THP1 + PMA	Vit D + LPS +THP1	Vit D + LPS + THP1+ 2.5µg Fonio
D	10 µg Fonio +THP1	10 µg Fonio +THP1 + Vit D	10µg Fonio +THP1 + RA	10µg Fonio +THP1 + PMA	Vit D + RA + PMA +THP1	Vit D + RA + PMA + THP1+ 2.5µg Fonio

NB. NBT was not performed for all wells of the above experiment that was repeated 4 times, due to lack of NBT reagents (lack of funding). But all images of the THP1 cells during the experiment were taken and saved.

Statistical Analysis: SPSS version 18 was used for the analysis. A One-Way ANOVA between groups was performed followed by Turkey's Multiple Comparison.

3. Results

*3.1 Beta-glucan extraction.*The modified Carr method (1990) used for extraction of beta-glucan from fonio flour yielded a good concentration after freeze-drying (60%). Fonio, alike many cereals has a low lipid content [3]. Therefore, the treatment with ethanol (refluxing) in order to defat high fat samples before the alkaline extraction of beta-glucan,

was unnecessary. Two types of extracts were obtained after neutralizing with

hydrochloride acid; a brown fraction with a pH of 7.4 and a gray fraction with a pH of 7.2

that was used for the micro and macroculture of the THP1 cells (closest to neutral pH 7).

*3.2 Summary of data obtained from CD1 study.***Table 3** summaries data obtained from

the CD1 pilot study. Apart from the body weights, there were significant differences

between all measured parameters. The mice treated with fonio extracts produced higher

levels of the inflammatory cytokines in vivo (serum) or in vitro (spleens) tests. Spleen

weights of the treatment group were also higher than those of the control group.

Table 3: CD1 mice body, spleen weights and selected cytokines production.

	CD1 ctrl	CD1 Fonio Trt	*p ≤
Body weight (g)	21.26 ± .55	21.74 ±0.39	.497
Rel. Spleen weight (mg/g)BW	4.46 ± .394^b	6.53 ± .240^a	.001
Spleen MTT (OD)	0.333 ± .0391^b	0.345 ± .0073^a	0.04
Spleen TNF-α (pg)	15.67 ± 2.45^b	269.20 ± 38.18^a	0.02
Spleen IL-6 (pg)	18.47 ± 10.57^b	136.09 ± 20.13^a	.002
Serum TNF-α (pg)	31.75 ± 19.96^b	909.56 ± 53.58^a	.000
Serum IL-6 (pg)	23.85 ± 5.43^b	638.43 ± 122.56^a	0.037

Values are mean ± SEM. Means in rows followed by different superscript letters are significantly different; a>b; p <0.05.

3.3 Cell proliferation and viability assessed by the MTT Test. In **Figure 2,** the MTT

results showed that all concentrations of the fonio extracts significantly reduced the

proliferation of THP1 cells as compared to the baseline. These results were obtained from
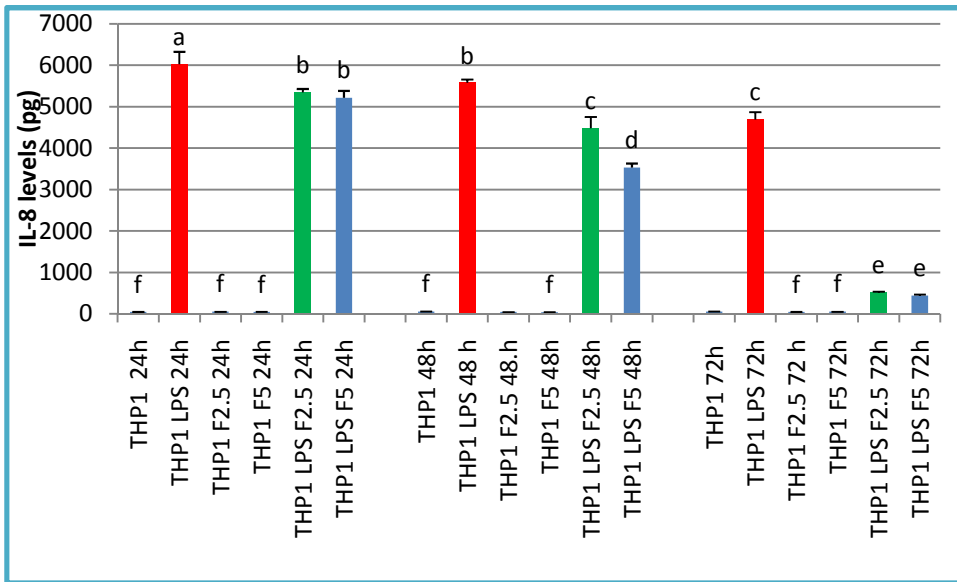
3 experiments run in triplicates.
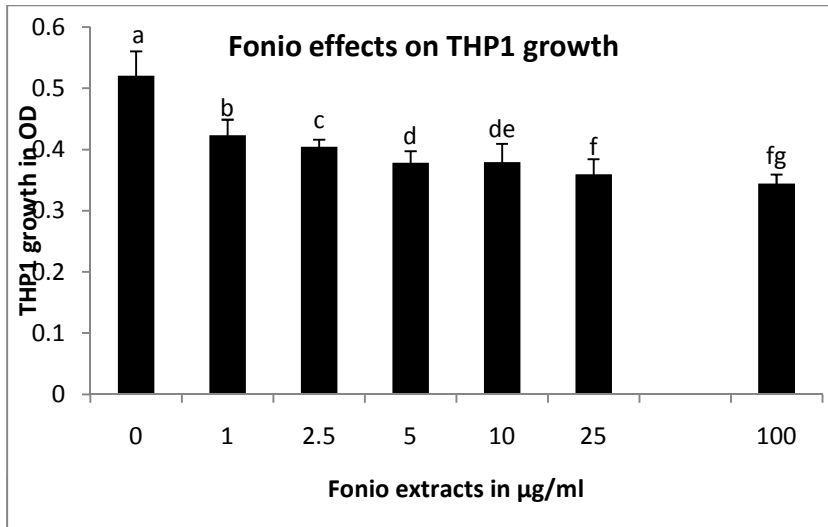
Bars with different letters are significantly different; a>b>c; p <0.05.

Figure 2: MTT results from THP1 incubated with different fonio extracts concentrations.

In **figure 2**, fonio extracts significantly decreased THP1 cells proliferation in a dose dependent manner from concentrations between 1 and 5µg, but concentrations of 5 and 10 µg were not different as well as concentrations of 25 and 100 µg. Therefore, only concentrations of 2.5 and 5 µg of extracts were used for the macroculture to test for their effects on inflammatory cytokines.

3.4 IL-8 and IL-6 production of THP1 treated by the combination of LPS and fonio extracts

Figure 3 shows that challenging cells with LPS resulted in elevated amounts of IL-8 secreted by THP1cells without fonio extracts (6028, 5579 and 4693 pg) during all three time points (24, 48, and 72 hrs, respectively). However, the different treatments with 2.5 and 5 µg of fonio extracts significantly reduced IL-8 expression in a timely dependent manner (P = .001).

Bars with different letters are significantly different; a>b>c; p <0.05.

Figure 3: IL-8 production of THP1 cells treated with LPS and fonio extracts.
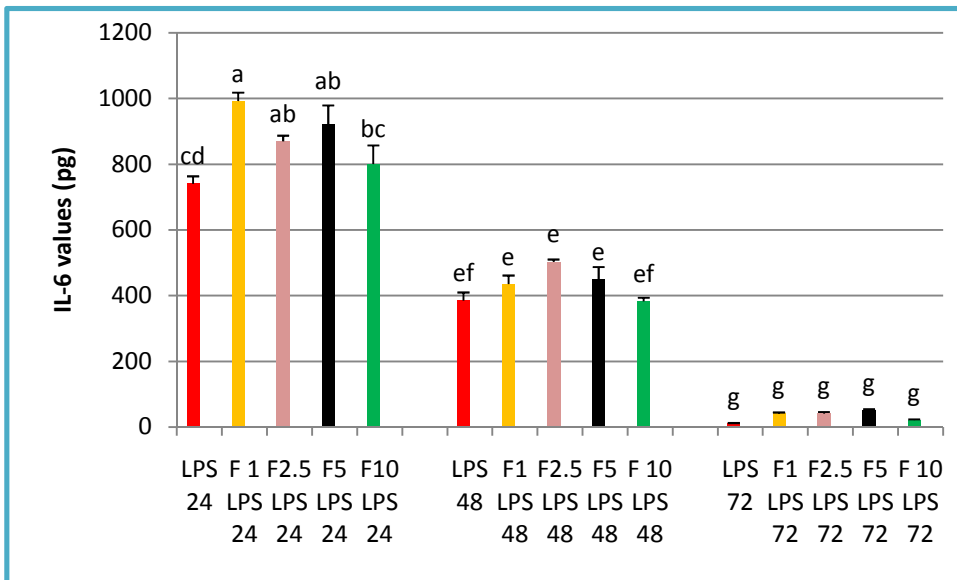
Bars with different letters are significantly different; a>b>c; p <0.05.

Figure 4: IL-6 production of THP1 cells treated with LPS and fonio extracts.

IL-6 values for THP1 alone with or without fonio were practically undetectable. After

being challenged with 2.5 µg LPS and treated with different concentrations of fonio (1;

2.5; 5; and 10 µg), results showed that there were high levels of IL-6 produced by THP1

cells treated with LPS (Mean = 740 Pg) that significantly decreased (P =0.0001) across

the set time points of 24, 48, and 72 hours (**Figure 4**). However, LPS challenged THP1

cells treated with 1, 2.5 and 5µg of fonio extracts, produced significantly higher levels of

IL-6 than those treated with LPS alone at 24 hours (P= 0.0001, 0.047, 0.001,

respectively); after 48 and 72 hours, they were no longer different. There was no

difference between LPS challenged THP1cells and those treated with LPS and 10 µg

fonio extracts at any time period. Compared to IL-8 results, opposite effects were

obtained with IL-6 secretions after LPS challenge. It is interesting to note that the lowest

fonio concentration (1µg) yielded the highest levels of IL-6, although not significantly

different to 2.5 or 5 µg.

3.5 Dye exclusion and NBT testing for cell apoptosis or proliferation. We conducted the

dye exclusion test to determine whether fonio extracts exerted cytotoxic effects on THP1

cells to retard or stop growth. Results showed in **Table 4** show that cells conserved their

viability. We used cell viability of 85% and above for all our experiments. Therefore, we

concluded that fonio used mechanisms different from apoptosis to decrease growth rate.

Table 4: Viability of THP1cells treated with fonio extracts using the dye exclusion test.

Fonio extracts	0 µg	1 µg	2.5 µg	5µg	10µg
Viability (%) Ep1	90.56	92.00	91.21	92.06	90.66
Viability (%) Ep2	85.67	94.87	93.57	93.57	95.25
Viability (%) Ep2	94.30	91.43	91.32	90.54	92.98
Means ± SEM	90.18 ± 2.50	92.77 ±1.06	92.03 ±0.77	92.05 ±0.87	92.9 5± 2.30

The next step was to determine whether fonio extracts decreased growth rate via cell differentiation. For that purpose we conducted the NBT test. This test clearly differentiates between myeloid cells and mature cells. Premature cells cannot reduce nitrozolium blue dye into black-colored formazan salts. The test is very accurate when monocytes differentiate into macrophages; after reduction of the nitrozolium salt, the obtained black formazan is sequestered inside the macrophages, granulocytes and other cells that will further be dissolved in DMSO and ODs are measured at 540nm.

Figure 5 shows results obtained from THP1 after they were incubated for 5 days with and without the fonio extracts. Fonio extracts induced differentiation (confirmed by NBT results) in a dose dependent manner (**Figures 5**) but without apparent morphological changes (**Figure 5A and 5B**).
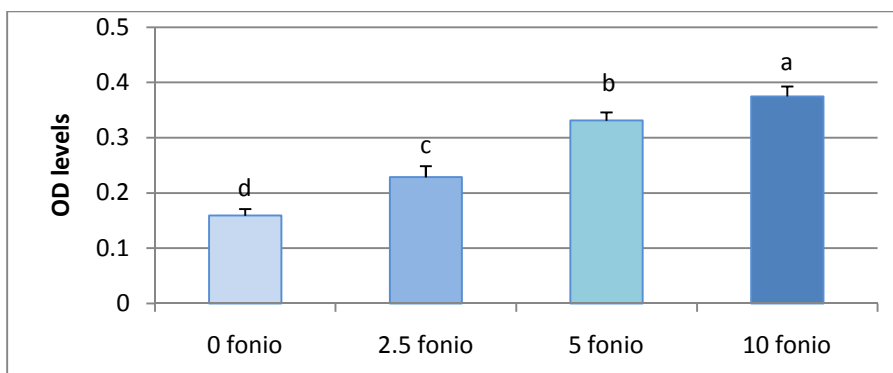
Bars with different letters are significantly different; a>b>c; p <0.05.

Figure 5: NBT test of THP1 cells treated only with different concentrations of fonio extracts. These data are means obtained from 4 experiments.

Table 5 shows the results obtained from the different combinations of THP1 cells, all trans retinoic acid (ATRA), Vitamin D and phorbol myristate acetate (PMA) with or without fonio extracts. THP1 cells were completely differentiated by ATRA alone although cells visibly started to undergo necrosis (fuzzy cell membrane) [**Figure 7A**].

Table 5: NBT for THP1 cells with Vit D, ATRA or PMA with or without fonio extracts.

	0 µg fonio	2.5 µg fonio	P* $\leq$
Vit D (OD)	0.309 ±0.015	0.450± 0.021	0.001
ATRA (OD)	0.246 ±0.026	0.333± 0.014	0.003
Vit D/ATRA (OD)	0.125± 0.033	0..316± 0.030	0.001
Vit D/ ATRA/ PMA (OD)	0.227± 0.027	0.382± 0.024	0.001

THP1 cell differentiation was better achieved with the combination of ATRA and Vitamin D, but necrosis visibly started at day 5, recognizable by the slightly damaged cell membranes (**Figures 8A);** when fonio extracts were added, the new differentiated cells proliferated more since there were much more cells absorbing the formazan salt (ODs raising from 0.125 up to 0.316); also there are visibly more new cells in the image where fonio extracts were present) and viability was also enhanced (cells membranes were

100

finely defined) [**Figure 8B**]. Similar results were obtained from the combination of ATRA/VITD/PMA and ATRA/VITD/PMA/fonio; the presence of fonio extracts induced proliferation and enhanced viability of the new cells with ODs raising from 0.227 to 0.382 (**9A and 9B**) and pictures visibly showing a higher density of new cells with the fonio extracts .

Both ATRA and vitamin D are known for inducing differentiation, whereas PMA is a strong mutagen to induce cell proliferation. They are widely used in NBT techniques to induce cell differentiation (uptake of formazan salt) first, and then the compound to be tested is added. If uptake of the formazan salt is increased, then differentiation capacity is attributed to the tested compound (Makashima et al 2003; Komoura et al 2007; Chen et al 2009; James et al 1997).
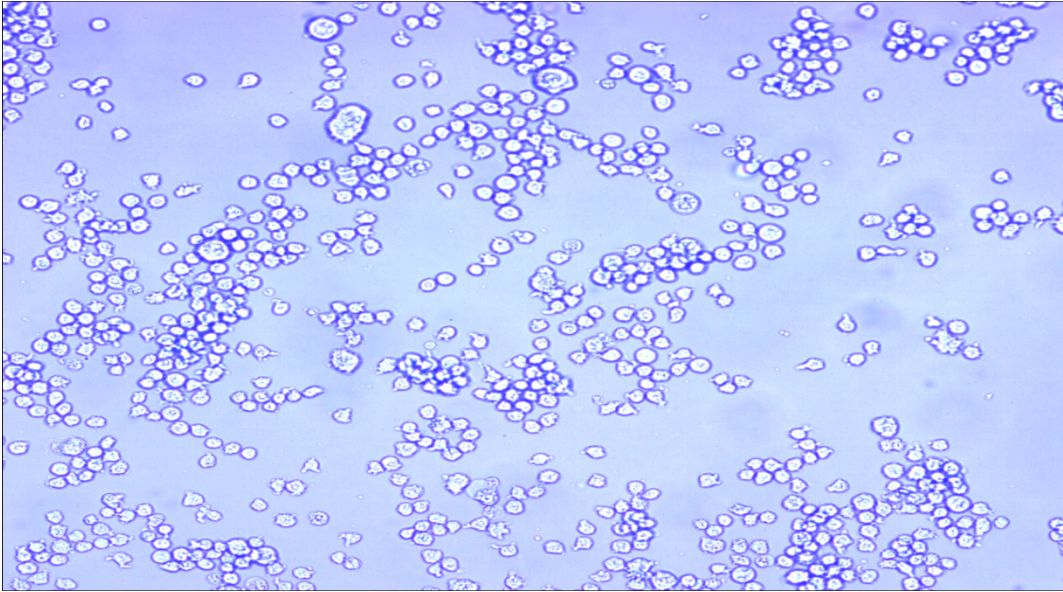
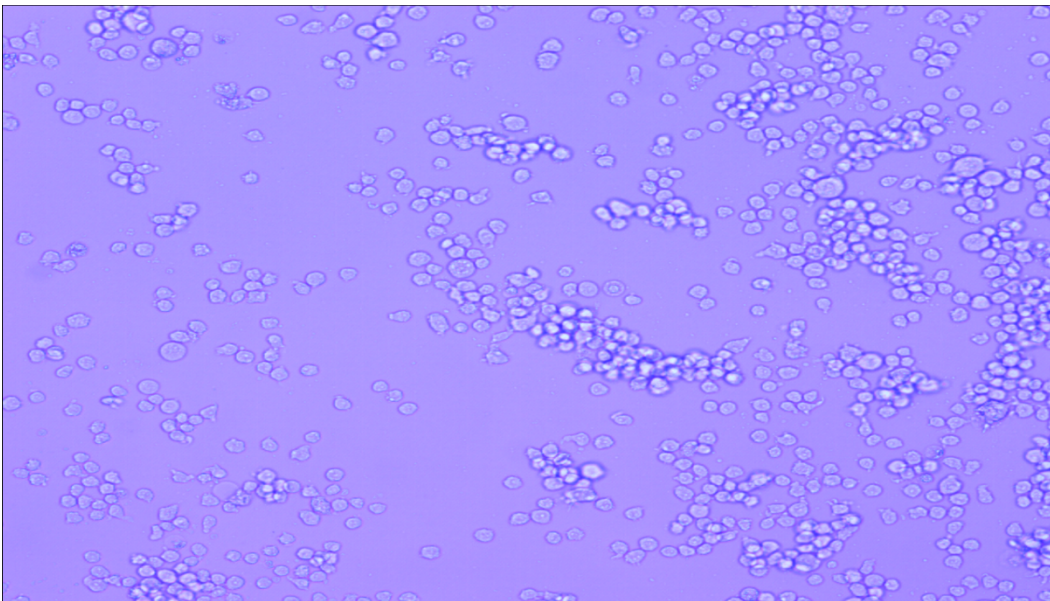**Figure 5A.** THP1 Cells without fonio extracts after 5 days of incubation

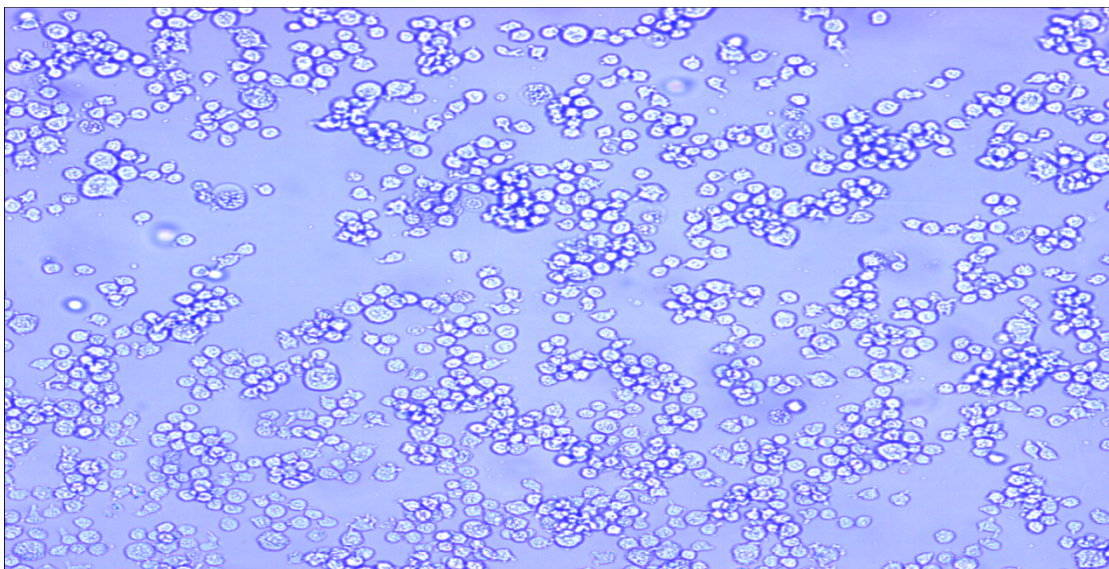**Figure 5B**: THP1 Cells with fonio extracts after 5 days of incubation

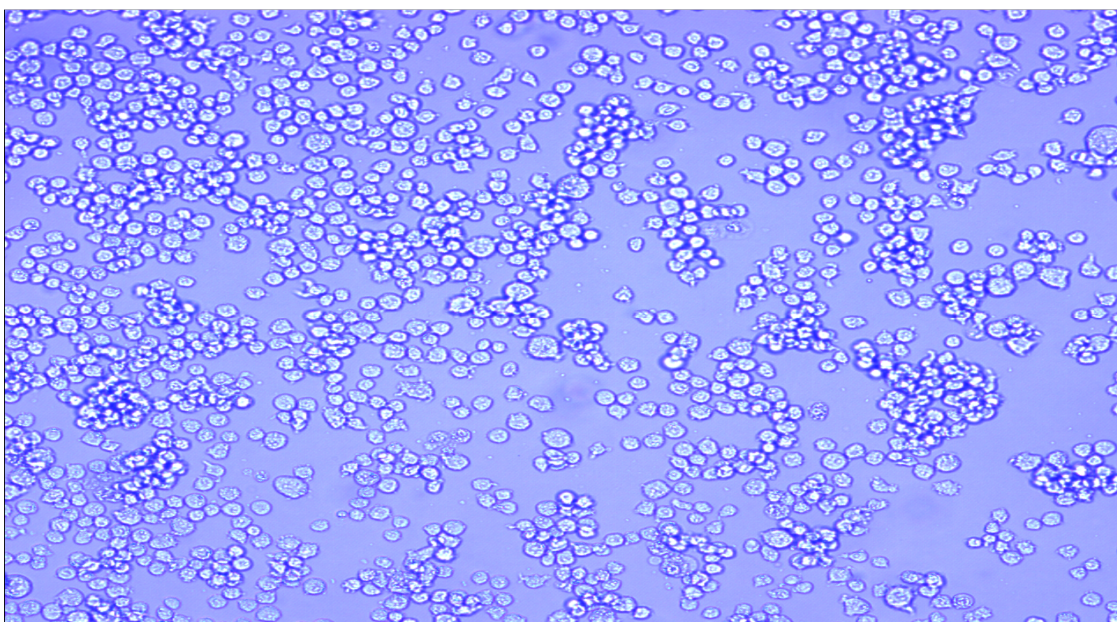**Figure 6 A**: THP1 cells incubated with Vit D for 5 days, without fonio extracts

Figure 6B. THP1 cells incubated with Vit D for 5 days, with fonio extracts

After 5 days of incubation with Vit D, THP1 cells alone underwent slight morphological changes. Those changes were enhanced when the cells were incubated with fonio extracts. The observations were confirmed by NBT results in Table3.

Figure 7A. THP1 cells incubated with ATRA for 5 days, without fonio extracts

Figure 7B. THP1 cells incubated with ATRA for 5 days, with fonio extracts

After 5 days of incubation with ATRA alone, THP1 cells underwent important morphological changes (Fig 6A). Those changes were enhanced when the cells were incubated with ATRA and fonio extracts (Fig 6B). The observations were confirmed by NBT results in Table3
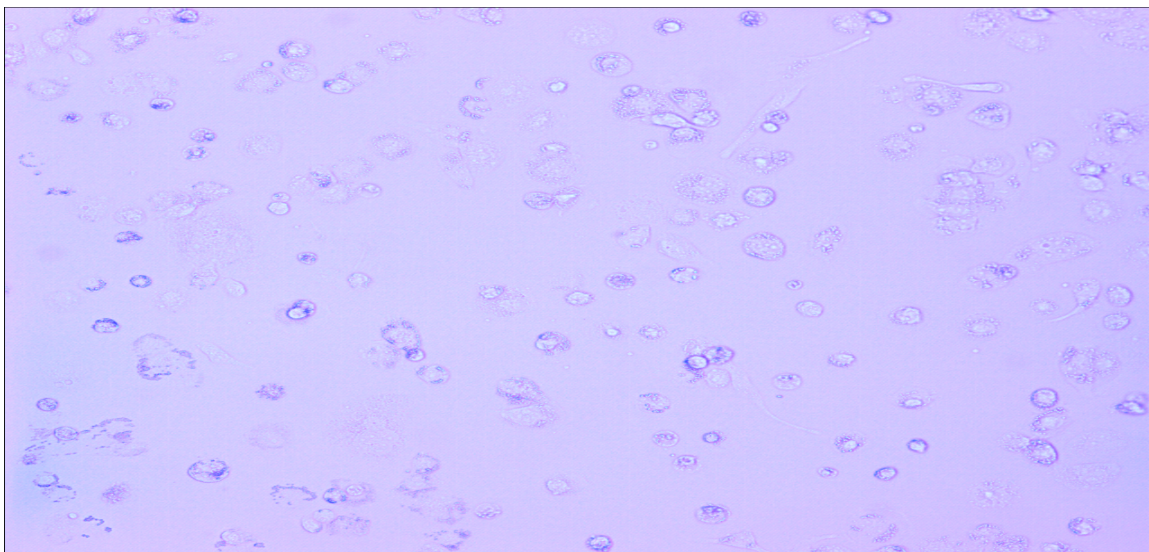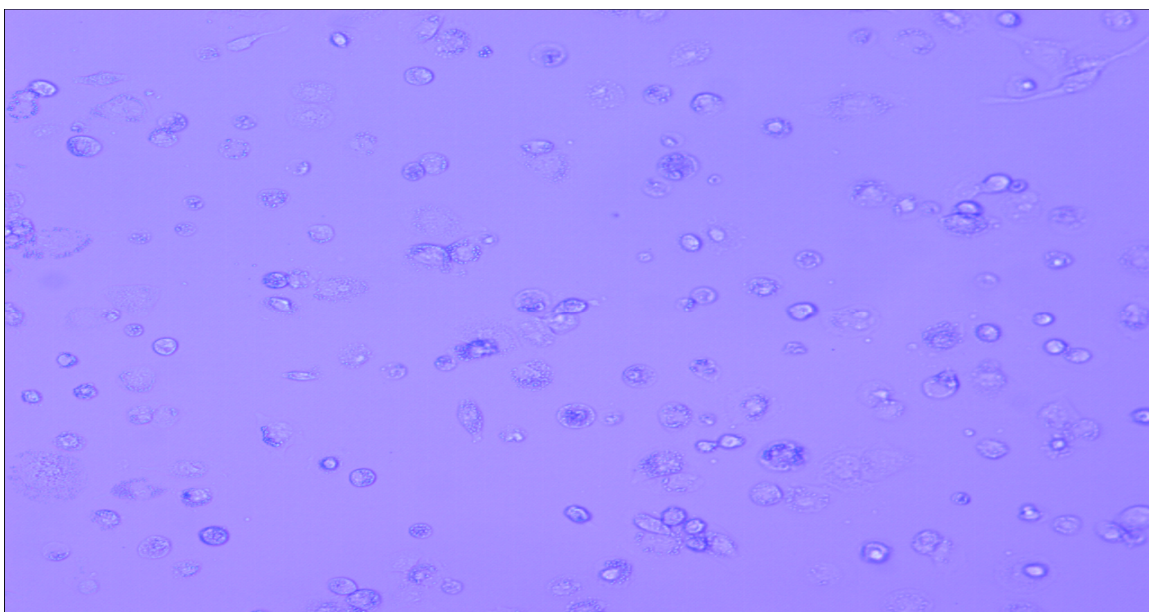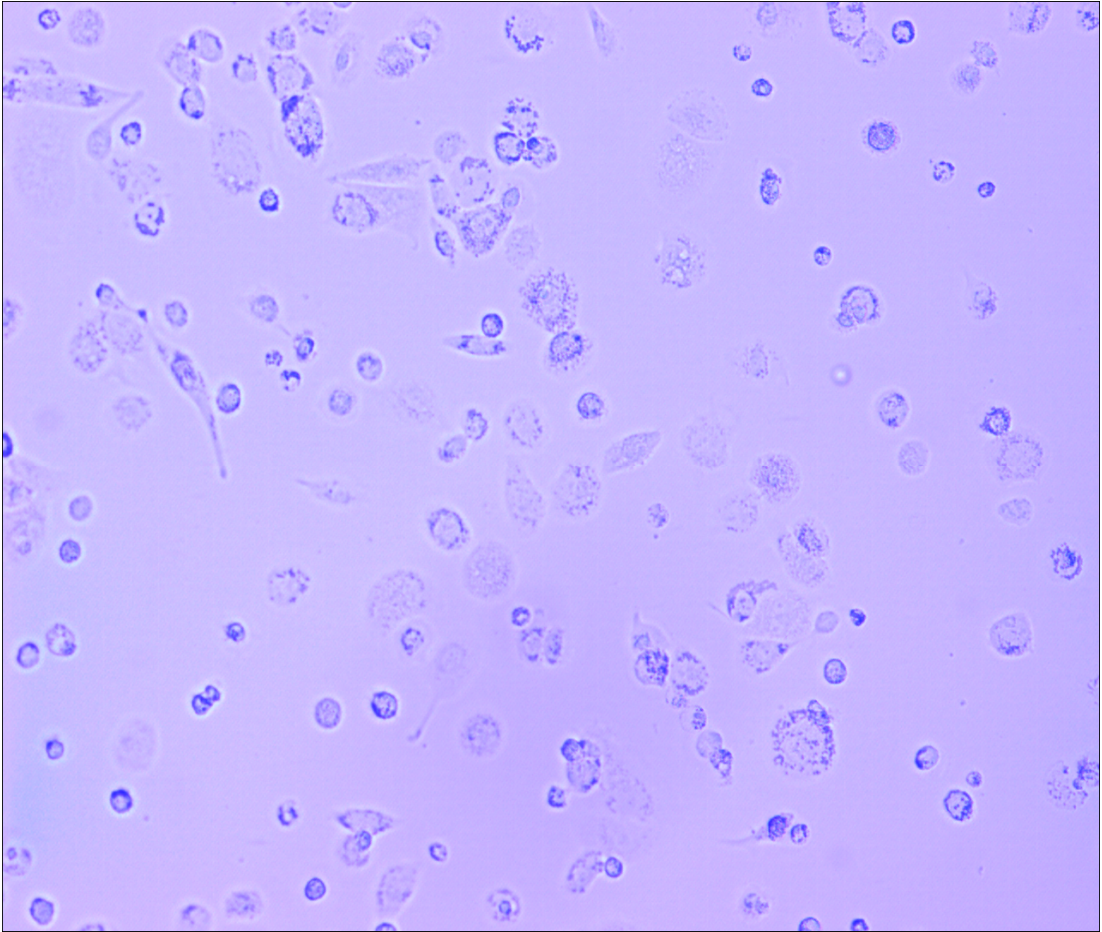
Figure 8A: THP1 cells incubated with Vit D and ATRA for 5 days, without fonio extracts
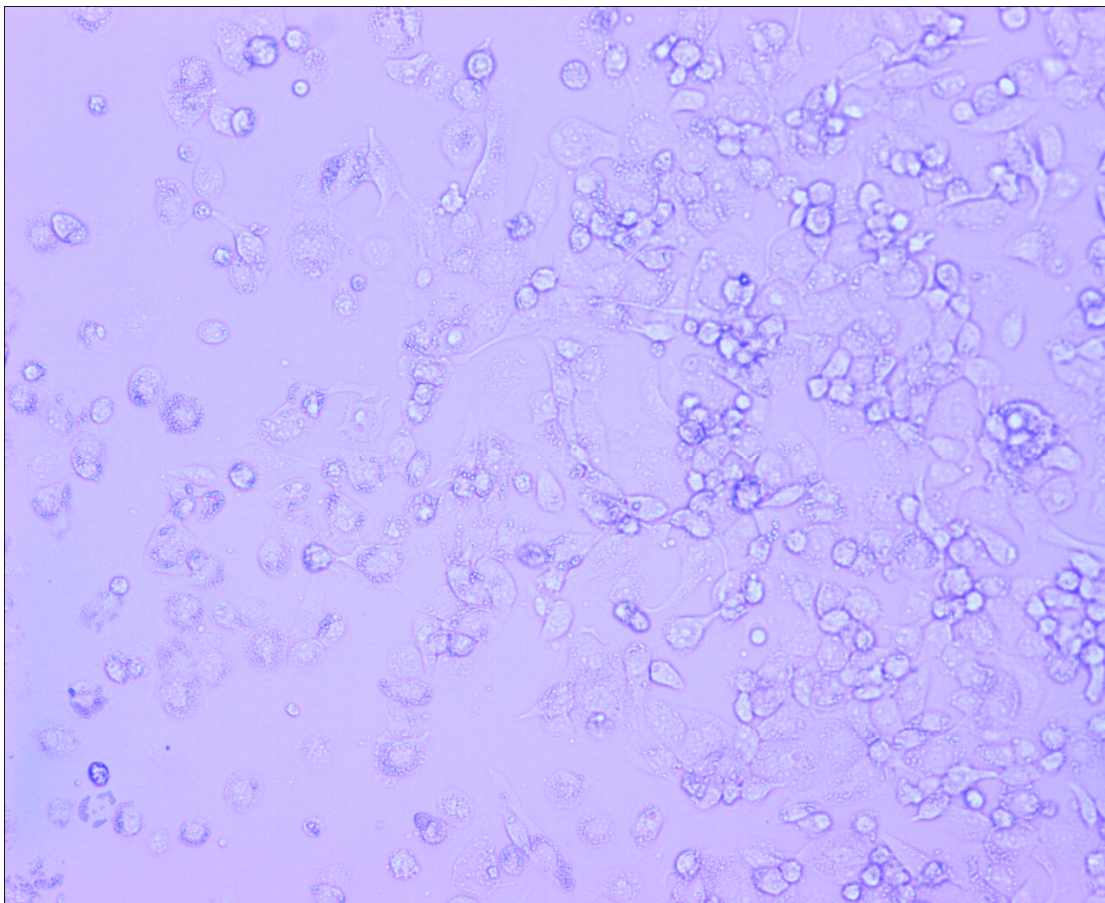
Figure 8B: THP1 cells incubated with Vit D and ATRA for 5 days, with fonio extracts

When cells were incubated with both Vit and ATRA (**Figure 6A**), 2 observations could be made: both differentiation and apoptosis took place resulting in fewer but completely different cells. When cells were incubated with Vit D, ATRA and fonio extracts **(figure 6B)**, 2 observations were made: differentiation and proliferation of the new cells. Clusters of new cells could be observed**.** Observations are confirmed by NBT results in Table3
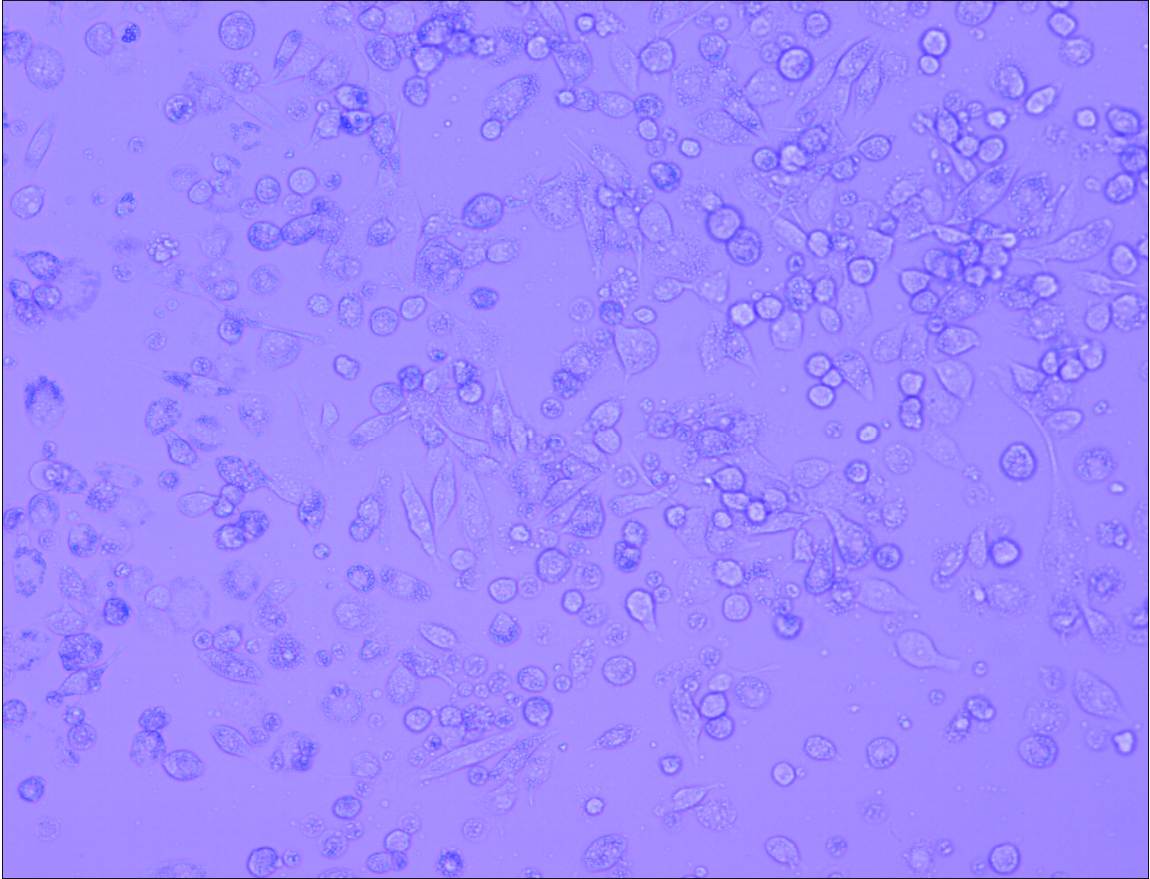
Figure 9A: THP1 cells incubated with Vit D, ATRA and PMA, for 5 days without fonio

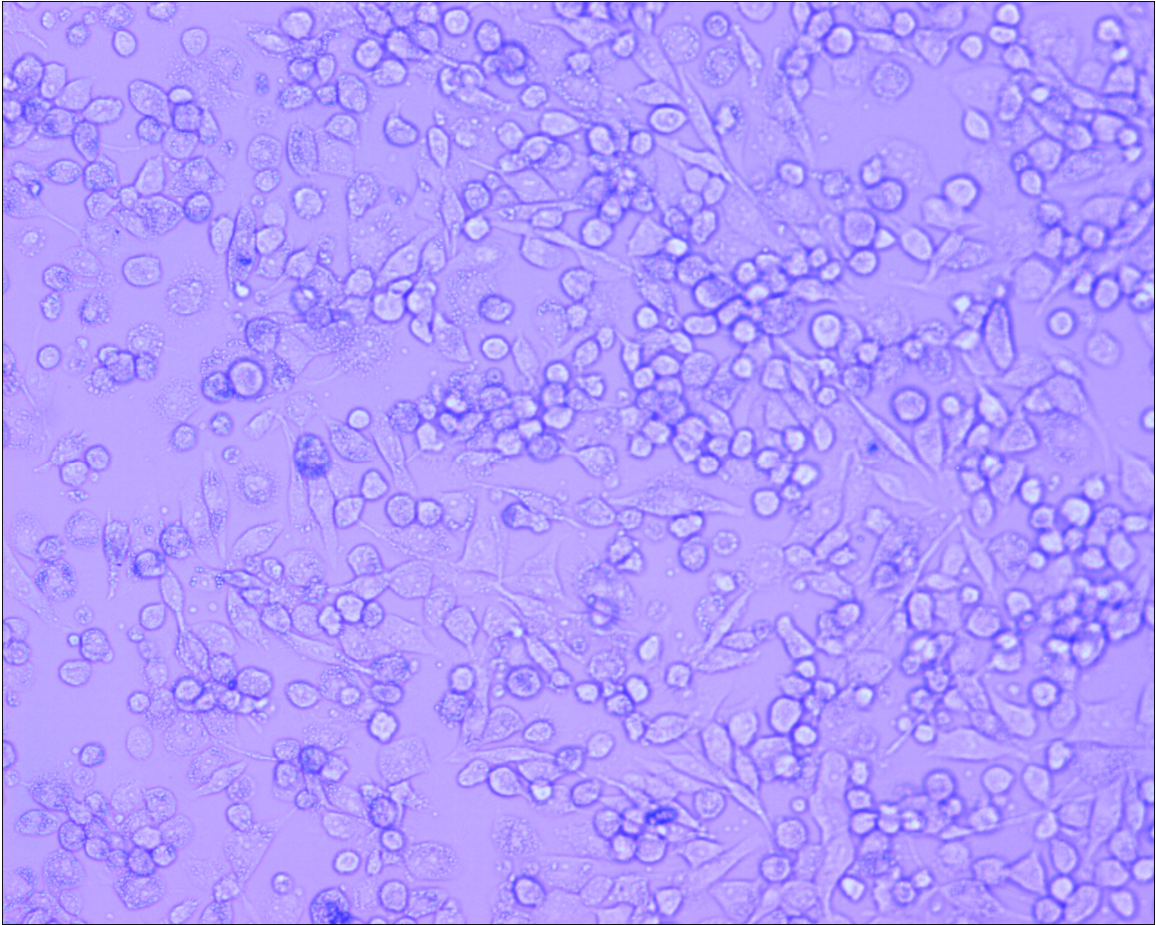**Figure 9B:** THP1 cells incubated with Vit D, ATRA and PMA, for 5 days, with fonio

Experiments described in figures 6 A and B, were replicated but in the presence of PMA, a strong mitogen; the same results were obtained with more cells with or without fonio. More clusters are observed with the fonio treatment. Observations are confirmed by NBT results in Table 1.

3. Discussion

The fonio extracts-treated CD1 mice. After acute inflammation was induced in the CD1 mice with 5 micrograms of LPS for two hours, the fonio-fed group expressed higher levels of IL-6 as compared to the control group (P=0.037), suggesting a successful initiation of the immune system, powered by T-lymphocytes and macrophages. IL-6 is known to be central to the immune system in response to inflammation and infection (van der Poll et al 1997) and is produced by T-lymphocytes and macrophages (Grimble 1996). The cytokine plays a major role for the survival of host cells during acute inflammation survival, keeping them from undergoing apoptosis (Hodge et al 2005). And during infection, Ishihara and his colleagues (2003) showed that macrophages secrete quantities of IL-6 which induce intracellular signaling cascades that further lead to the production of other inflammatory cytokines. The same effects were observed in our study where IL-6 production was accompanied with high levels TNF-alpha. The control group failed to respond after LPS challenge since they produced significantly lower levels of IL-6 and TNF alpha (p= 0.037 and P=0.0001, respectively).

Similar results were obtained in vitro: after CD1mice spleens were treated with 1 microgram LPS, those from the fonio-fed group produced significantly higher levels of IL-6 and TNF-alpha (P= 0.002 and P= 0.02, respectively). In addition, their spleen weights (fonio-fed group) were significantly heavier than those of the control group (P= 0.001) after 2 hours of the LPS challenged, suggesting a higher priming rate of naïve T-lymphocytes and more recruitment of new cells. Interestingly, the best results (highest levels of pro-inflammatory cytokines) were obtained with the lowest concentrations of fonio extracts (1 µg) for both in vivo (serum) and in vitro (spleen) experiments.

Therefore, fonio extracts have immune stimulatory effects and can be an excellent candidate as a functional food against infection.

Another important observation was made with the CD1 mice during the first hour of the treatments: both control and treatments groups were moving very slowly but in the following hour, the fonio group started moving faster. The same observations were made in humans; four clinical studies demonstrated that pre-treatment of high-risk surgical patients with poly-[1-6]--D-glucopyranosyl-[1-3]--D-glucopyranose glucan (PGG-glucan) supplied intravenously decreased the infection incidence and need for antibiotics, shortened intensive care unit length stay, and ultimately improved survival compared to a saline placebo injection (Babineau et al 1994; Babineau et al 1994; Browder et al 1990, Dellinger et al 2003). Also, in all the above cited studies, patients of the treatment group had significantly higher serum pro-inflammatory cytokines.

THP1 cells treated with fonio extracts. MTT Results showed a progressive reduction of THP1 proliferation in a dose-dependent manner. The mechanisms by which the reduction took place were investigated. For that purpose, the dye exclusion test was performed and results showed that in all tested fonio extracts concentrationsTHP1 cells conserved their viability above 85 % suggesting that reduction of proliferation was not apoptotic or necrotic and that extract did not exert cytotoxic activity.

After the first 24 hours of incubation, reductions of 11.22 and 13.46 % in IL-8 expression were observed with the treatments of 2.5 and 5 µg respectively. At 48 hours, there were reductions of 20 and 37 %, but at 72 hours there were drastic reductions of approximately 89 and 91% in IL-8 expression. Interestingly, when inflammation was not present, fonio extracts did not affect THP1 cells secretion of the cytokine since there were no significant

differences between the amounts of IL-8 produced by the THP1 cells alone and those of the THP cells treated with fonio alone. This observation was in agreement with the study of Brown and Gordon (2003) who noted that low molecular weight beta-glucans would modulate the response of cells only when they are stimulated by an inflammatory environment, contrary to high molecular weight beta-glucans. Although this study focused on inflammation, the decrease of IL-8 induced by the extracts may lead to a new line of research on cancers. IL-8 was found to be involved in growth and invasiveness of estrogen-negative breast cancer cells and promoted tumor (Freund et al 2003).

The NBT test clearly showed that the mechanism by which fonio decreased cell proliferation was cell differentiation. When cells were treated with fonio extracts alone, the differentiation was initiated, confirmed by the results of the NBT although there was not a visible morphological change. Vitamin D and ATRA are known to induce cell differentiation. Several studies used this method to assess the capacity of a compound to differentiate cells (Makashima et al 2003; Komoura et al 2007; Chen et al 2009; James et al 1997). Cells are first incubated with ATRA alone or with its combination with Vit D and the ODs are recorded. Then the compound to be tested is incubated with ATRA or Vit D or with the combination; if a higher OD is obtained then it is concluded that the compound is involved in differentiation. Recently, Nurath and Finotto (2011) showed that IL-6 signaling was crucial for T-lymphocytes differentiation and activation. Although THP1 cells are monocytes, we can infer from the study of Nurath and Finnotto that the presence of high levels of IL-6 may explain one process through which differentiation of the monocytes (THP1) occurs. They recently showed that IL-6

signaling was crucial for T-lymphocytes differentiation and activation (Nurath and Finotto 2011).

As we stated above, NBT tests implied that fonio extracts alone induced THP1 cells differentiation in a dose-dependent manner (figure 4), but without visible morphological changes. The best compound that was seen to differentiate THP1 alone was ATRA, or its combinations with other compounds. But for unknown reasons, necrosis in THP1 cells started to take place with ATRA and its combinations at day 5. However, the major finding of this study is the capability of the fonio extracts to inhibit necrosis and to enhance differentiation. In all ATRA treatments, fonio extracts kept the viability of the new differentiated cells and allowed their proliferation. These properties can be visually observed when comparing figures 8A and 8B, or 9A and 9B and they are confirmed by the NBT results (0.125 against 0.316 and 0.227 against 0.328, respectively).

In summary, the following are the new findings of the current study:

a. Fonio extracts raised inflammatory cytokines in CD1 mice after inflammation was induced by LPS.

b. The extracts decreased THP1 proliferation

c. After LPS challenge, fonio-treated THP1 increased IL-6 levels but interestingly reduced IL-8 levels.

d. Cell differentiation is the mechanism by which fonio decreased THP1 cell differention

e. The combination of ATRA and fonio extracts completely differentiated the monocytes (THP1) into macrophages and other granulocytes.

The limitations of our study are: It would've been interesting to run flow cytometry in order to determine the new cell strains after differentiation occurred.

4. **Conclusion**

Fonio extracts revealed a potent immuno-modulatory capacity. Just one microgram triggered strong effects in both spleen cells of CD1 mice and did the same with the THP1 cells. In addition, fonio could stop proliferation rate of the THP1, a human cancer cell line by inducing their differentiation into macrophages, fibroblasts and granulocytes. In addition, the extracts drastically reduced IL-8 secretion in THP1 cells. IL-8. Those characteristics could pave new lines of research for the wild cereal.

Implications/recommendations. The extracts of fonio could be used as an alternative to HIV patients of whom, the impaired immune system exposes them to many infections. Those patients took lots of antibiotics with so many adverse effects. A functional food made of the extracts may drastically reduce their antibiotics intake. Also, the extracts may be a serious candidate against breast cancer.

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CHAPTER VI

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary of findings

The major objectives of these studies were to find out whether or not *Digitaria exilis* (fonio), a wild cereal mainly found in West Africa had the alleged traditional anti-diabetic properties that local populations believed in. For that purpose two studies were undertaken: in the first one, AIN 93 (regular mice feed) was supplemented with fonio or rice to test their effects on KK/HIJ mice; in the second study, THP1 cells and normal CD1 mice were treated with fonio extracts to investigate its effects on inflammation and the mechanisms involved. After completion of the studies, the main findings are summarized below:

a. After four months of dietary treatment, results showed that supplementation the diet with fonio can improve insulin resistance and glucose tolerance in KK/HIJ mice.

b. Feed intake in the fonio-fed group was significantly reduced as compared to the rice and control groups.

c. Supplementing the KK/HIJ mice with fonio blocked the development of their yellow color characteristic of the expression of the yellow agouti gene (one gene of obesity).

d. Fonio decreased plasma levels of IL-6, but not TNF-α. Decreased IL-6 will modulate blood levels of C-reactive protein, a marker of inflammation and risk factor of complications of chronic diseases including type-2 diabetes.

e. Compared to rice, fonio also tended to decrease in vitro secretion of IL-1β and IL-6, but not TNF-alpha by LPS activated spleen cells. IL-1β is known to induce apoptosis in cells of islets of Langerhans.

f. Fonio extracts raised inflammatory cytokines in CD1 mice after inflammation was induced by LPS.

g. Fonio extracts decreased THP1 proliferation

h. After LPS challenge, THP1 cells treated with fonio extracts responded by expressing higher levels of IL-6, but interestingly, they produced lower levels of IL-8 when compared to controls.

i. Cell differentiation but not necrosis, was the mechanism by which fonio decreased THP1 cell proliferation.

j. The combination of ATRA and fonio extracts completely differentiated the monocytes (THP1) into macrophages and other granulocytes. This differentiation was further enhanced by vitamin D.

Conclusions

In conclusion, data suggest that fonio may modulate diabetes severity by reducing inflammatory cytokines and increasing insulin sensitivity. Supplementation with fonio blocked the development of the characteristic yellow fur of KK/HIJ mice, suggesting the down-regulation of the adult-onset yellow agouti gene. Fonio extracts revealed a potent immuno-modulatory capacity: just one microgram triggered strong effects in both spleen

cells of CD1 mice and THP1 cells. Even though our studies were confined to investigating fonio effects on diabetes only and not cancer, it was found that fonio extracts could reduce proliferation rate of THP1 cells, a human cancer cell line by inducing their differentiation into macrophages (fibroblasts and granulocytes). In addition, the extracts drastically reduced IL-8 secretion in THP1 cells. Studies have demonstrated a strong involvement of IL-8 in breast cancer. Those characteristics could pave new lines of cancer research with the wild cereal.

Implications/Recommendations

a. A functional food made of fonio/fonio extracts should be developed and its effects tested on obese, pre-diabetic and diabetic people.

b. The functional food/fonio extracts could be used as a supplement for HIV patients whose impaired immune system exposes them to many infections. HIV patients, contrary to healthy people, express low levels of pro-inflammatory cytokines when they are infected with pathogens. Therefore, during infection, those patients are given high doses of antibiotics with so many adverse effects. A functional food/fonio extracts may drastically reduce their antibiotics need.

c. Because of the fact that fonio extracts reduced the proliferation of THP1 cancer cell line, and decreased IL-8 production, their effects should be tested against breast cancer.

Limitations

The limitations of our study were:

    a. KK/HIJ mice were received by groups of 10 over a 2-month period forcing us into a slight modification of our design and protocol. After receiving the first 10 mice, 4 were assigned to the AIN diet; 3 to the fonio-supplemented diet; and 3 to the

rice-supplemented diet. A month later, we received 10 more mice and 4 were

assigned to the fonio diet and 3 to each of the other 2 diets; and so on with the

following third group of 10 mice received (4 assigned to rice-supplemented

diet…etc.). Some of the mice were received in fall and some at the beginning of

spring; treatments in different seasons may generate biases.

b. Although we had 3 groups of 20/diet, we further subdivided them in two sub-

groups to test the effects of short versus longer exposure to the diets. Therefore

some tests (like GTT) lacked of statistical power.

c. It would have been interesting to run flow cytometry in order to determine the

new cell strains after differentiation occurred.

d. Important tests such as quantification of the yellow obese gene/mRNA after

treatment were crucial but because of lack of enough funding we couldn't.

CHAPTER VII

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Appendix 1: Cell Proliferation

Reagents and materials and required:

Mediums, solutions, & special reagents Major sources (vendors) of these reagents and supplies include Sigma, Invitrogen/GIBCO, Fisher, BD Biosciences, and VWR.

Fonio extracts : stock solution containing 1 mg/ml (refer to Annex 4 for extract preparation)

Mitogens: Lipopolysaccharides (LPS)

Concentration of stock solution: 100 μg/ml

Use 10 μL/1 ml cell suspension (the equivalent of 1 μg/ml

Or perform mitogen dose response before the actually study of interest. Based on past experience, the optimal concentration is between *1 and 5, quite often 2.5 μg/ml. Levels ≥7.5 μg/ml are inhibitory.*

MTT reagent (Source Sigma): dissolve to obtain 5 mg/ml PBS.

Filter the sample and use 25 μL/well.

Keep at 4°C protected from light by wrapping the tube with aluminum foil.

MTT is stable for 2 weeks (even longer) as long as it is not contaminated with cells.

Isopranol – 0.08N HCl: solution to stop the MTT reaction: Use 50 μL/well (96 well-plates).

Culture medium: composed of RPMI-1640, 25 mM HEPES, 2 mmol/l L- glutamine, 0.1 mmol/l non essential amino acids, 1 mmol/l sodium pyruvate, 50 μmol/l β-

mercaptoethanol, 50-100 international units/ml (50,000-100,000 international units/l) penicillin, and 50-100 μg/ml (50-100 mg/l) streptomycin (P/S).

Wash medium: PBS supplemented with either 0.5% or 1% BSA (bovine serum albumin), or 1% FCS (fetal calf serum; fetal bovine serum), and 50-100 Units/ml penicillin, 50-100 μg/ml streptomycin (P/S).

Red blood cells (RBCS) lysing buffer: *sterile ice cold distilled water* or a filtered solution of ammonium chloride (NH_4Cl, 8.3 g), potassium bicarbonate ($KHCO_3$, 1.0 g), and EDTA (ethylene diamine tetra-acetic acid) 1.8 ml of 5% stock solution) dissolved in 1000 ml deionized water).

Trypan blue- 0.4% for viable cell count

Drabkin's reagent (reagent available)

Kits: IL-6, IL-8, (or others based on Specific Aim of the experiment)

Sterile 2 cc, 5 cc, and 10 cc serological pipettes.

Pasteur pipettes; pipetors or pipet-aid

Bench paper

Gloves
Sterile blue tips (1000 μL capacity); yellow tips (2-200 μL capacity)

200 cc capacity beaker with about 100 ml 70% ethanol

200 cc capacity beaker with saline (0.85-0.9% sodium chloride solution) to wash tissues such as liver, heart, kidney and lungs (tissues that may contain a lot of blood). *Note: if sodium chloride interferes with assays to be performed on these tissues, saline shall not be used.*

Surgical supplies (scissors, forceps)

CO2, ether, isoflurane, or any other suitable (approved) chemicals for anesthetizing mice;

24 well plates (for macrocultures); 96-well plates (for cell proliferation/microculture)

Dry ice or liquid nitrogen (if tissues need to be frozen immediately)

Scintillation vials – 7 ml capacity with 2 ml formalin: 1/mouse (if tissues or bones have to be fixed immediately for future histology study)

Dissecting board

Balance

Water bath

Refrigerated centrifuge machine (capable of achieving 500-6000 rpm or at least about 400 x g)

Tubes & other supplies:

12 x 75 mm (5 ml capacity) for Hb assay: 2/mouse (2.5 ml Drabkins reagent/tube)

2 ml Cryovials (for freezing leftover cells): 2/mouse – 1/spleen, 1/thymus – if required

Hematocrit (Hct) tubes & clay (sealant – sealing material)

15 ml conical centrifuge tubes (sterile) with 10 ml PBS: 2/mouse (1/Spleen, 1/thymus)

Cell strainers: 2/mouse (1 for spleen & 1 /thymus)

Two 3 cc or 1 cc capacity syringes (no needles): 1/spleen; 1/thymus

CO_2 incubator set at 37°C & 5% CO_2: Important note: To control humidity, water must be put in a dish in the lower shelf of the incubator.

12 x 75 mm (5 ml capacity): 1/spleen; sterile, with 1 ml PBS

12 x 75 mm (5 ml capacity): 1/thymus; sterile, with 1 ml PBS

1.5 ml capacity eppendorf tube: 1 for each tissue/mouse [heart (H); liver (L); kidney (K); LU (lungs)];

1.5 ml capacity Eppendorf tube for blood sample: 1/mouse

1.5 ml capacity eppendorf tube for collecting plasma: 1/mouse

400 μL or 1.5 ml capacity eppendorf tube: 1/spleen cell counts

400 μL or 1.5 ml capacity eppendorf tube: 1/mouse for WBC (white blood cells) counts

400 μL or 1.5 ml capacity eppendorf tubes: 1/mouse for thymocyte counts

Composition of some reagents

Beta-mercapto-ethanol:

Stock solution A: To make 1 M, mix 0.5 ml with 6.6 ml distilled water.

Stock solution B: To make 50 mM, mix 5 ml of stock solution A with 95 ml distilled water

For culture medium requiring 50 μM β-mercaptoethanol, add 100 μL to 99.9 ml of culture medium (final volume 100 ml medium).

Composition of phosphate buffered saline (PBS) 1X (working solution):

0.23 g NaH2PO4 (anhydrous) (1.9 mM)

1.15 g Na2H PO4 (anhydrous) (8.1 mM)

9 g NaCl (154 mM)

Add 900 ml distilled deionized water

Dissolve all salts

Adjust pH to 7.4 by adding drops of either 1M NaOH or 1M HCl

Bring total volume to 1000 ml by adding more deionized water

Add fetal calf serum (FCS) and antibiotics (P/S, 50-100 units (add 1-4% glucose as desired)

Important note: PBS is sterilized by filtration but not autoclaving.

Appendix 2: **Mice treatment**

Anesthetize the mouse by CO2, ether, or isoflurane inhalation

Weigh the mouse and record the info on the chart

Draw as much blood as possible by retro-orbital plexus or other methods and transfer to heparin containing 1.5 ml conical tubes. If serum is desired, transfer the blood to a tube without anticoagulant.

Important Note: To limit hemolysis, keep tubes at room temperature.

Centrifuge vials or tubes no later than 60 min.

Collect plasma or serum and transfer to new 1.5 ml eppendorf vials (tubes) and freeze at -80°C until used for various assays.

If the volume allows, the plasma/serum can be split in 2 trace elements, etc) have to be done at different times.

After drawing blood, "flood - wash" the mouse abdomen with 70% EtOL

Remove the spleen under sterile conditions and transfer immediately to a 12 x 75 mm culture tube containing 1 mL PBS (sterile).

Weigh immediately and transfer the tube to 4°C (ice bucket).

NOTE: Never leave spleen in a tube without PBS or medium because it will undergo autolysis.

Remove the thymus also under sterile conditions and transfer to sterile 12 x 75 mm tube, weigh, and transfer to 4°C (ice bucket).

Remove the liver, heart, kidneys, and lungs and transfer each to pre-weighed 1.5 ml capacity eppendorf tubes. (Rinse extra blood in saline or distilled water.)

Weigh and immediately transfer to liquid nitrogen or dry ice.

If required, cut one or both front and back legs, remove skin and muscle and freeze in liquid nitrogen or at -80°C. Legs may also be frozen with skin and muscle. Tibia and femurs may also be collected and transferred to formalin for bone studies.

Preparation of Spleen Single cell Suspension: Except with centrifugation, all steps are to be performed under a tissue culture hood.

Prepare single cell suspension by standard techniques:

Gently grind the spleen on a sterile cell strainer placed on a 40 cc capacity beaker or a 50 cc capacity centrifuge tube with use of the inner portion of a 1 cc or 3 cc syringe.

Wash the cell strainer with 10 ml PBS and collect the fluid and transfer to a 15 ml conical sterile centrifuge tube.

Centrifuge tubes at 1200 rpm, 4°C for 10 min.

Decant

Resuspend the cell pellet in 1 ml, sterile ice cold deionized water

Mix the cell pellet by inverting tube or shaking (Do not vortex). This step must be done rapidly if not cells will form clumps.

Incubate tubes on ice for 20-60 sec (average 30 sec). Incubation time varies with the size of the pellet. But never leave cells in water for more than 60 sec at a time.

Fill the tube with 10 ml ice cold PBS supplemented with either 0.5-1% BSA or FCS.

Mix rapidly and centrifuge as explained under Step 11.

Note: To avoid in vitro iron repletion, high concentration of FCS (>2.5%) should not be used.

Alternative methods:

Resuspend pellet in 2 ml ammonium chloride solution

Incubate at room temperature for 5 min

Add 8 ml of wash (PBS/FCS/P/S) medium to each tube

Centrifuge at 4°C, 400 x g or 1200 rpm for 10 min.

Decant.

If the cell pellet is still red, repeat Steps 13-17.

Otherwise, resuspend the cell pellet in 1-3 ml culture medium.

Important notes: 2.5% FCS supplemented culture medium is recommended when dealing with spleens from iron deficient animals. However, when iron or other trace elements present in serum are not variables under study, 10% FCS supplemented medium is recommended.

Mix the cell pellet either by shaking the tubes or with a pipet.

To avoid clumping, this step must be done very rapidly.

To perform total and viable cell counts, combine 10 μL of cell suspension and 90 μL of 0.4% trypan blue (or 20 μl of cell suspension and 180 μl trypan blue in an eppendorf tube. (This is the equivalent of 1:10 dilution).

Transfer cells to a haemocytometer.

Count the number of dead (blue) and live (clear) cells in the center square and/or center plus 4 corners of one side of the hemocytometer.

Calculate viable cell concentration as follows:

N x 106 / ml = (number of viable cells counted   x 106

Number of squares counted x 10

If a 1: 20 dilution was done, the cell concentration obtained above should be multiplied by 2.

If a 1:5 dilution was made (10 µL cells plus 40 µL trypan blue or 20 µL plus 80 µL), the cell concentration should be multiplied by 0.5 (or divided by 2).

If cells were counted only in one square, the denominator will be 1 x 10.

Appendix 3: Macrocultures for secretion

Transfer 2 x 106 viable cells to each well on a 24-well plate (4 cultures/spleen) according to the chart shown below.

Add 10 µL, 25 µL, 50 µL, 100 µL mitogens (Con A, PWM, LPS, sterile stock solution of 100 µg/ml), or culture medium (for non-activated cells).

Bring total volumes to 1 ml with complete RPMI-1640-25 mM HEPES medium (see composition page 1).

The final concentration of mitogens is 1 µg/ml, 2.5 µg/ml, 5 µg/ml, and 10 µg/ml when 10 µL, 25 µL, 50 µL, 100 µL of stock solutions were used, respectively.

IMPORTANT NOTE: Mitogen Dose Response is done only with Preliminary Experiment or when a New Lot # of a Mitogen is received. For The purpose of the experiment, you shall perform a mitogen dose response!

After mixing cells by tilting the plate, transfer 24-well plates to a CO_2 incubator set at 37°, 5% CO_2, in a humidified atmosphere.

After 24-72 h of incubation, centrifuge the plates at about 900-1000 rpm, 4°C, for 10 min.

Collect the supernatant and transfer aliquots (about 500 μL) to 1.5 ml capacity eppendorf vials.

Since cytokine secretion is time dependent and varies with the type of cytokine, it may be a good idea to perform a time dependent response (8 h, 12 h, 24 h, 48 h, or 72 h.

Important Note: Incubate plates for 72 and Record the information in your research notebook!

Vials need not be sterile but must be clean and not previously used.

Vials with supernatants must be kept on ice or cold blue box.

Care must be taken not to mix the cell pellet because the pellets will affect cytokine levels or volume at the time of cytokine assay.

Freeze supernatants at -80°C until needed for cytokine and immunoglobulin assays.

Cell Proliferation by Either MTT Test or 3H-thymidine Uptake.

Label sterile several tubes per spleen (12 x 75 mm culture tubes). The # of tubes needed depends on the number of cultures or mitogen doses to be tested.

Transfer 1 x 106 viable cells to each.

Do not add any mitogen to tube 1: baseline or non-activated cells.

Add 10 μL, 25 μL, 50 μL, 100 μL mitogens (Con A, PWM, LPS, sterile stock solution of 100 μg/ml) to tubes a to X.

Bring total volume per tube to 1000 μL by adding culture medium.

For baseline culture, also add medium to up to 1000 μL.

Mix cells and mitogens by shaking the tubes – DO NOT VORTEX.

Transfer 200 μL of cells to 3 wells.

Incubate plates in CO2 incubator, 5% CO2, 37°C, in a humidified atmosphere.

After 68 h, add 25 μL of 5 mg/ml MTT sterile solution (pre-warmed at 37°C for 5 min or left at room temperature for 15-30 min protected from light).

Important Note: Turn the light off in the tissue culture hood while adding MTT solution to wells.

2.40 ml of MTT are needed per 96-well plate. To be on a safe side, for each 96-well plate, remove 3.0 ml of MTT from the flask and transfer to a new small tube for immediate use.

To avoid contamination, Do not return used MTT to the original container or flask.

Also to avoid cross contamination, use 1 pipet tip per well or use 8-channel pipet for fast delivery of solution.

For MTT blank, transfer 200 μL of culture medium (no cells) to a set of 12 wells and incubate the plate in parallel to the microcultures. Add MTT and/or isopropanol-HCl solution as for microcultures.

To avoid waste of 96-well plates, one can also transfer 400 μL culture medium to 3-4 12 x 75 mm tubes.

At appropriate times, add 50 μL MTT to each tube and 100 μL isopropanol-HCl solution. Then mix the tubes and transfer 275 μL to 1 well/tube on a non-sterile but clean 96-well plate.

The reading of OD is done as for microcultures.

Cover the plate with a piece of aluminum foil.

After 4 h of further incubation in CO_2 incubator, add 50 μL isopropanol-HCL solution to each well.

Seal the plate with transparent paper and read optical density (OD) according to protocol (570 nm with a reference wavelength of 650nm. (Some investigators have also used 540 nm for OD determination.)

Calculate the mean OD of triplicate wells and the ratio of OD obtained with mitogen over baseline (culture without mitogen).

Express the results as percent of background.

Cryo-preservation of unused cells.

Prepare freezing medium by adding 0.5 ml of DMSO (dimethyl sulfoxide) to 9.5 ml culture medium. Filter the medium and keep on ice.

Note: Freezing medium is prepared just before use and shall not be stored for more than 60 min.

Centrifuge remaining spleen single cell suspensions at 1200 rpm, 4°C for 10 min.

Resuspend the cell pellet in 1-2 ml or about 5 x 106 cells/vial (sterile cryovial, 2 ml capacity).

Transfer the cells into sterile cryovials and incubate at on ice for 30 min.

Transfer to liquid nitrogen immediately or -80°C overnight but not longer. If spleen cells are kept at -80°C for more than 24 h, cell viability significantly decreases.

Appendix 4: Oklahoma State University Institutional Animal Care and Use Committee

From: IACUC
Sent: Tuesday, August 11, 2009 3:12 PM
To: Kuvibidila, Solo
Subject: ACUP HE-09-4 (Kuvibidila) Approved

Oklahoma State University
Institutional Animal Care and Use Committee (IACUC)

Protocol expires: 8/10/2012

Date: Tuesday, August 11, 2009   Animal Care and Use Protocol (ACUP) No : HE-09-4

Proposal Title: Effects of Fonio, Sorghum and Rice on the Pathogenesis of Diabetes in KK Mice

Principal
Investigator:

Solo Kuvibidila
Nutritional Sciences
301 HES
Campus

Reviewed and   Full Committee
Processed as:

Approval Status Recommended by Reviewer(s):   Approved

The revised protocol is approved as written. You are approved for 140 KK/HIJ 002106 mice for the next three years.

Signatures :

Tuesday, August 11, 2009

Charlotte Ownby, IACUC Chair   Date

cc: Department Head,   Nutritional Sciences
        Director, Animal Resources

Approvals are valid for three calendar years, after which time a request for renewal must be submitted. Any modifications to th
research project, course, or testing procedure must be submitted for review and approval by the IACUC, prior to initiating any
changes. Modifications do not affect the original approval period. Approved projects are subject to monitoring by the IACUC.
 OSU is a USDA registered research facility and maintains an Animal Welfare Assurance document with the Public Health Servi
Office of Laboratory Animal Welfare, Assurance number AA3722-01.

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VITA

Djibril Traore

Candidate for the Degree of

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Major Field: Nutritional Sciences

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Pages in Study: 146

Candidate for the Degree of Doctor of

Philosophy

Major Field: Nutritional Sciences

Scope and Method of Study. *Digitaria exilis* (fonio), a wild cereal consumed in West Africa, is rich in chromium, β-glucans and sulfur amino acids, factors known to modulate inflammation and diabetes. However the mechanisms are unknown. We hypothesized that fonio might: a. modulate diabetes pathogenesis through improvement of insulin resistance, glucose metabolism, and reduced inflammation; b. trigger the inflammatory response during infection; c. induce differentiation of immune cells to modulate overall immunity. To test our hypothesis, 3 studies were conducted: *In sudy 1*, 4-week old KK/HIJ transgenic male mice were fed either the AIN 93 diet or the same diet fortified with 30% fonio or rice flours (n=20). Ten mice per dietary treatment group were sacrificed after 3 or 4 months of feeding. Glucose and insulin tolerance tests (GTT & ITT) were studied by standard techniques. *In study 2*, non diabetic healthy mice CD1 mice were challenged with bacterial lipopolisaccharides (LPS) and treated with fonio extracts and were sacrified two hours later. To assess the effects of fonio extracts on in vitro secretion by immune cells, spleen cell suspensions $(2 \times 10^6/ml)$ were activated with 2.5 µg/ml LPS for 48 h (study 1 and 2). *In study 3,* we investigated the mechanisms by which fonio extracts modulate cytokines secretion. For that purpose, monocytic THP1 cells were incubated without and with different concentrations of fonio extracts for 24-72 h. Cell proliferation, viability and differentiation were assessed by trypan blue exclusion test, nitroblue tetrazolium salt reduction, and microscopy, respectively. Cytokines in plasma and spleen cell supernants from the 3 studies were measured by enzyme immune-absorbent.

Findings and Conclusions. (1) fonio improved insulin resistance and reduced low-grade inflammatory cytokines in KK/HIJ mice. (2) Fonio extracts significantly up-regulated the secretion of TNF-α (pro-inflammatory), and IL-6 (immune modulatory-pro-inflammatory) secretion *in vivo* and *in vitro* in LPS-treated mice. (3) Fonio extracts reduced monocytic THP1 cell growth via differentiation, and not necrosis. This is the first study showing that fonio can modulate the secretion of inflammatory cytokines in transgenic and non-diabetic mice. The current data will allow us to conduct a translational human study.

ADVISER'S APPROVAL: <u>Dr Solo Kuvibidila</u>