ALTERATIONS IN INDICES OF OXIDATIVE STRESS AND DIABETES IN TYPE I DIABETIC RATS ON A LOW-CARBOHYDRATE DIET AND PYCNOGENOL

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ABSTRACT

Glucose, the major fuel for most body tissues, derives largely from ingested carbohydrates. Considering that a primary feature of diabetes is persistent hyperglycemia, reducing exogenous carbohydrates is expected to moderate absolute serum glucose values and oscillations. Chronically elevated glucose levels are known to increase oxidative stress in tissues amongst other effects, and lead to late and chronic complications in kidney, eye and the cardiovascular system. The objective of this study was to evaluate the impact of a very low-carbohydrate diet alone or with addition of pycnogenol, a potent antioxidant, on biomarkers of oxidative stress in various body organs as well as on physiological indices of diabetes in a Type 1 diabetes (streptozotocin induced) model. In diabetic rats at both 30 and 90 days, the low-carbohydrate diet (27% protein, 5-12% carbohydrate, 62-68% fat) significantly reduced blood glucose, glycated hemoglobin, alanine aminotransferase and normalized aspartate aminotransferase, implying less damage to tissues than a standard rat chow (27% protein, 61% carbohydrate, 12% fat). The test diet greatly minimized polyuria, polydipsia, polyphagia and loose stool in diabetics. It further improved fasting triglycerides and HDL cholesterol, blood urea nitrogen and creatinine. In both studies, diabetes-induced alterations in cardiac catalase and glutathione peroxidase, hepatic γ-glutamyl transpeptidase and erythrocyte malondialdehyde were ameliorated or normalized by the test diet. Addition of pycnogenol reduced serum alkaline phosphatase, triglycerides, total cholesterol, liver γ-glutamyl transpeptidase, renal glutathione peroxidase and renal reduced glutathione. It increased cardiac and renal glutathione to oxidized glutathione ratio. In non-diabetics the

low-carbohydrate diet elevated serum alkaline phosphatase, total and HDL cholesterol and reduced water intake. It reduced liver and kidney catalase. Pycnogenol had beneficial effects on blood malondialdehyde and glutathione molecules, and increased retinal glutathione peroxidase and reductase. Pycnogenol lowered total and HDL cholesterol, and decreased liver and renal catalase. Overall, the low-carbohydrate diet ameliorated several factors altered by diabetes and did not engender worse effects. The benefits may have resulted from lowered glucose levels or from effects of dietary fatty acids. Addition of pycnogenol vastly improved the health status of diabetic animals in particular. Pycnogenol had mixed effects in the non-diabetic kidney that warrant further investigation.

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Abbreviations

Abbreviation	Definition
A/G	albumin to globulin ratio
AGEs	advanced glycated end products
ALEs	advanced lipo-oxidation end products
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
ASCP	American Society of Clinical Pathologists
AST	aspartate aminotransferase
B/C ratio	blood urea nitrogen / creatinine ratio
BUN	blood urea nitrogen
CAT	catalase
DCCT	Diabetes Control and Complications Trial
DM	diabetes mellitus
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	disodium ethylenediamine tetraacetate
eNOS	endothelial nitric oxide synthase
GGT	gamma glutamyl transpeptidase
GLUT 2	glucose transporter 2

GPx	glutathione peroxidase
GRx	glutathione reductase
GSC	gamma glutamyl cysteine ligase
GSH	glutathione (reduced form)
GSSG	glutathione (oxidized form)
HbA_{1c}	glycated hemoglobin
HDL	high density lipoprotein cholesterol
4-HNE	4-hydroxynonenal
IgG (H+L)	immunoglobulin G (heavy and light chains)
LC diet	low carbohydrate diet
LDL	low density lipoprotein cholesterol
M2VP	1-methyl-2-vinylpyridinium triflouromethanesulfonate
MDA	malondialdehyde
NADH	nicotinamide adenine dinucleotide
NADP+	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NEM-	N- ethylmaleimide
NO	nitric oxide
NOS	nitric oxide synthase
OPT	*o*-phthaladehyde
PKC	protein kinase C
Pyc	pycnogenol
ROH	water or alcohol

ROOH	an organic peroxide
ROS	reactive oxygen species
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOD	superoxide dismutase
STZ	streptozotocin
TBS	Tris – buffered saline
TBS-T	Tris – buffered saline with 0.05% Tween 20
UKPDS	United Kingdom Prospective Diabetes Study
UV	ultraviolet light

CHAPTER ONE

INTRODUCTION

1.1 Inspiration

An increased awareness of obesity and the need for effective and feasible weight loss formulas has popularized the low-carbohydrate diets that are alleged to facilitate rapid weight loss with minimal discomfort of hunger. An analysis of several studies comparing low-carbohydrate diets and the standard high carbohydrate diets was not able garner enough evidence to advocate or condemn either one (Bravata et al., 2003). A few endocrinologists have suggested the low-carbohydrate high-fat diet to their patients (Bernstein, 1997). This study was prompted by a student who is diabetic and had been placed on a very low-carbohydrate diet to help maintain a constant and normal glucose control. In a preliminary clinical study (both type I and type II diabetics, some on insulin) whereby glucose concentration was rigorously regulated with the insulin regimen strictly tailored to requirements, and where carbohydrates were restricted to a total of 30gm a day, both glycated hemoglobin (HbA_{1c}) and lipid profile (increased high density lipoprotein cholesterol (HDL), and decreased triglycerides, low density lipoprotein cholesterol (LDL) and total cholesterol to HDL) improved significantly compared to baseline values (O'Neill, 2004) demonstrating that a low-carbohydrate diet has some desirable effects. Knowing that chronically elevated blood glucose present in subjects with diabetes mellitus leads to an increase in oxidative stress, an objective of this study

was to find out how this very low-carbohydrate diet impacts on antioxidants in various body organs in order to ascertain whether such a diet would stem the development of late complications of diabetes.

1.2 Diabetes definition and statistics

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized chiefly by chronic hyperglycemia resulting from either an absolute lack of insulin, typical of Type 1 diabetes, or resistance of cells to the action of insulin and an inadequate compensatory increase in insulin secretion, a hallmark of Type 2 diabetes (ADA, 2004a). Type I DM results from an autoimmune destruction of ß-cells of the pancreatic islets of Langerhans in the setting of certain genotypes, and accelerated by viruses or other environmental factors. Subjects with type I DM have an absolute requirement for insulin. Type 2 DM develops from a resistance to insulin that is due to a combination of specific genetic makeup and environmental factors such as sedentary lifestyle and central obesity. These individuals benefit from physical exercise and dietary changes. A small percentage of diabetes mellitus is secondary to other disease processes such as pancreatic diseases, endocrinopathies and adverse effects of some drugs. The direct and indirect cost of DM in the USA was estimated at $132 billion for the year 2002 alone (ADA, 2002). Thus it is prudent to look for ways to alleviate this burden on individuals and the society at large.

1.3 Acute complications

Lack of insulin (the major anabolic hormone in the body) or resistance to it and unopposed glucagon create an imbalance in the metabolism of body fuels. Gluconeogenesis predominates in the liver and aggravates hyperglycemia. Greater breakdown of proteins and fats in muscle and adipose tissue cause muscle weakness, weight loss, and polyphagia. Glycosuria and polyuria ensue as plasma glucose levels exceed the renal threshold for reabsorption. The combination of loss of body water and high plasma osmolarity leads to polydipsia. With severe insulin lack, there is release of large amounts of free fatty acids and consequent formation of ketones beyond what the body is able to use. Dehydration limits the renal excretion of ketones resulting in ketonemia and ketoacidosis, a complication more prevalent in Type 1. Type 2 diabetics typically have enough insulin to prevent excessive release of fatty acids. These acute effects of chronic hyperglycemia are effectively managed by oral hypoglycemic agents, insulin, diet and exercise.

1.4 Insulin

Insulin and glucagon are fundamental regulators of carbohydrate, fat and protein use and storage in the body. Regulation of these macromolecules is needed to maintain physiological glucose levels since the brain, despite the fact that it can also use ketone

bodies as a source of energy, requires an absolute minimum level of glucose to function. Insulin secretion is stimulated largely by high glucose levels (after feeding) and its main functions are to increase the uptake of glucose into tissues (muscle and adipose), activate enzymes involved in storage of fuels, and inhibit enzymes that break down fuels. Consequently insulin deficiency or dysfunction affects the use of these macromolecules across the board being readily evident as the acute effects of DM as well as dyslipidemia. Excessive insulin leads to hypoglycemia and weight gain. Indeed in the Diabetes Control and Complications Trial (DCCT) where a standard amount of carbohydrate was being consumed (45-55% of daily caloric intake), the group on intensive control of glucose levels with insulin suffered three times the number of hypoglycemic events as the group on a standard insulin therapy (Lasker, 1993). A corollary of the low-carbohydrate diet maybe to reduce the amount of insulin required to achieve optimal blood glucose control.

1.5 Pathogenesis of chronic complications

Diabetes mellitus irrespective of the etiology is invariably accompanied by grave and chronic complications that become apparent several years after the onset of diabetes and greatly affect the length and quality of life. The principal damage is on blood vessels both small and large and manifests as retinopathy, nephropathy, neuropathy, and atherosclerosis and hypertension, respectively (Squadrito and Cucinotta, 1991).

The connection between hyperglycemia and the ensuing late complications is currently a rich field of research. Data from the Diabetes Control and Complications Trial (DCCT, Type 1 diabetes) (Eastman et al., 1993) and the United Kingdom Prospective

Diabetes Study (UKPDS, Type 2 diabetes) (Turner, 1998), demonstrated that strict control of blood glucose levels to as near the physiological value as possible slowed the onset and progression of these disorders. The findings support the hypothesis that a high blood glucose level is a prime source of these complications. At physiological levels, glucose is metabolized via a number of enzyme-catalyzed pathways, such as glycolysis, hexose-monophosphate shunt, storage as glycogen in muscles and the liver, and storage as triglycerides in adipose tissue and liver. Glucose can also participate in other reactions that become significant at abnormally high glucose levels. Dyslipidemia, commonly observed in diabetic subjects, is also a player in the genesis of the late complications. Several mechanisms linking high glucose levels to the late complications have been proposed (Figure 1.1). These include:

1.5.1. Glucose and generation of reactive oxygen intermediates

Oxidative phosphorylation in the mitochondria of aerobic organisms completely oxidizes glucose and generates superoxide anion in the process. Ordinarily the amount of superoxide anions produced is minimal and can be quenched ably by the reducing system of the cell. But in the high-in-glucose microenvironment of DM, superoxide anions rise to insurmountable levels and damage cellular contents and structures (Sheetz and King, 2002) as well as initiate a chain of reactions that generate other reactive oxygen species that are also injurious to cells. Glucose can also, in the presence of metal ions, undergo auto-oxidation to generate and propagate reactive species that similarly damage tissues (Giugliano et al., 1996b; Miyata et al., 1997). The reactive products of glucose auto-oxidation such as glyoxal may participate in glycation of macromolecules (see below)

and contribute to the abnormal induction of signal transduction pathways (Akhand et al., 1999).

1.5.2. Glycation and lipo-oxidation

Physiological post-translational modification of proteins includes the enzyme-catalyzed addition of a variety of sugars (glycosylation). Glycation, on the other hand, is the reaction whereby sugars such as glucose and fructose, and other alpha-oxoaldehydes and dicarbonyl compounds are spontaneously and non-enzymatically covalently bonded to free amino groups in proteins, lipoproteins and nucleic acids. Cross linking of glycated proteins leads to the formation of long-lived advanced glycation end products (AGEs) (Squadrito and Cucinotta, 1991). Glycation alters the structure, susceptibility to degradation, surface charge, interactions (e.g. increased aggregation), and functions of these proteins (Robertson et al., 2003; Thornalley, 2002). Targets for glycation include collagen, DNA, lipoproteins, membrane proteins, cytoskeleton, and plasma proteins (Thornalley, 2002). Glycated hemoglobin (HbA_{1c}) used to assess long-term glycemic control, is a well known example of a glycated product.

Metabolism of lipids is also deranged in diabetics as evidenced by the high triglyceridemia and undesirable lipoprotein fractions observed. Prevalence of oxidants in the hyperglycemic state favors the oxidation of these lipids as measured by lipoxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These lipo-oxidation products may also participate in chemically modifying proteins and their properties as the sugars do, to ultimately form advanced lipo-oxidation products (ALEs) (Januszewski et al., 2003). Both reactive intermediate carbonyl compounds and

AGE/ALE products may also directly attack target molecules, increase oxidative stress (Guzik et al., 2002), or act in a number of signal transduction cascades that eventually end in the abnormal expression of genes and tissue damage (Miyata et al., 2003; Miyata et al., 2000; Robertson et al., 2003).

AGE/ALE formation is a normal occurrence in tissues. The products are cleared from circulation via specific and non-specific receptors found on certain cells such as macrophages and endothelial cells (Thornalley, 1998). Inevitable but slow accumulation occurs with advancing age of an individual (Pageon and Asselineau, 2005). In DM and nephrogenic conditions (Henle and Miyata, 2003; Miyata et al., 2003) the rate at which these compounds are generated supersedes the elimination rate causing them to accumulate in and modify tissues (Squadrito and Cucinotta, 1991). The effect is particularly severe in certain tissues such as the mesangium of the kidney and basement membrane of vascular beds (Gugliucci., 2000), where they lead to local thickening, decreased elasticity and increased permeability (Squadrito and Cucinotta, 1991). These changes set the stage for subsequent alterations in hemodynamics such as increased blood flow and pressure to small vessels, and increased permeability to albumin and other plasma proteins that compound the microvascular damage (Squadrito and Cucinotta, 1991).

Glycation of ion pumps may contribute to an increase in osmotic stress and the formation of cataracts in diabetics (Stevens, 1998). Glycation of red blood cell membrane proteins reduces the cells' fluidity (Waczulikova et al., 2000) and ability to deform, and promotes the aggregation and increased viscosity of blood observed in DM subjects (Squadrito and Cucinotta, 1991).