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**DIETARY RESISTANT STARCH IMPROVED MATERNAL GLYCEMIC
CONTROL IN GOTO-KAKIZAKI RAT**

A Dissertation

**Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

In

The School of Human Ecology

By

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December 2010

DEDICATION

I would like to dedicate this to my major professor Dr. Martin and my family.

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TABLE OF CONTENTS

DEDICATION.....	ii
ACKNOWLEDGEMENTS.....	iii
LIST OF TABLES.....	vii
LIST OF FIGURES.....	viii
ABBREVIATIONS.....	ix
ABSTRACT.....	x
CHAPTER 1: INTRODUCTION.....	1
CHAPTER 2: REVIEW OF LITERATURE.....	4
2.1 Resistant Starch.....	4
2.2 Maternal Hyperglycemia.....	17
2.3 Beta Cell Dysfunction.....	28
2.4 Animal Models of Type 2 Diabetes	33
2.5 Methods of Measuring Insulin Sensitivity.....	44
2.6 Adiponectin.....	50
2.7 Physiological Role of UCP-1.....	54
2.8 Gut Microbiota and Metabolism.....	57
CHAPTER 3: DIETARY RESISTANT STARCH IMPROVES MATERNAL GLYCEMIC CONTROL IN GOTO-KAKIZAKI RAT	67
3.1 Introduction	67
3.2 Materials and Methods.....	67
3.3 Results.....	76
3.4 Discussion.....	87
CHAPTER 4: FEEDING DIETARY RESISTANT STARCH TO GOTO-KAKIZAKI RAT IMPROVES FASTING GLUCOSE IN OFFSPRING	91
4.1 Introduction	91
4.2 Materials and Methods.....	92
4.3 Results.....	95
4.4 Discussion.....	102
CHAPTER 5: CONCLUSIONS	105

REFERENCES.....	106
VITA.....	137

LIST OF TABLES

Table 2. 1 Summary of published human studies of glycemic index and Maternal Hyperglycemia.....	11
Table 2. 2 Comparison of normal pregnancy and gestational diabetes in terms of insulin resistance.....	21
Table 2. 3 Summary of animal models of type 2 diabetes.....	34
Table 3.1 Diet Compositions	69
Table 3.2 The sequences of primers for real time RT-PCR.....	74
Table 3.3 Food intakes and disemboweled body weight	86
Table 4.1 Food intakes and disemboweled body weight in offspring	101

LIST OF FIGURES

Figure 2.1 Mechanisms of glucose-induced insulin secretion	31
Figure 2.2 Normal shaped islet.	40
Figure 2. 3 Starfish shaped islet.....	40
Figure 2. 4 The phylogenetic tree of the most frequently detected phylotypes in human faeces using 16S rRNA gene sequencing	60
Figure 2.5 Distribution of microflora in gut	61
Figure 2.6 Proportion of Firmicutes and Bcteroidetes in lean and obese	63
Figure 3.1 Percentages of body fat/ disemboweled body weight	78
Figure 3.2 Fsting glucose concentration and HOMA-IR.....	79
Figure 3.3 Beta cell density and pancreatic insulin content.....	80
Figure 3.4 Cecal content PH and feces PH values	81
Figure 3.5 Bacteroides and Bifidobacterium population in cecum.....	82
Figure 3.6 Lactobacillus and Clostridial cluster IV population in cecum	83
Figure 3.7 Short chain fatty acids concentrations.....	84
Figure 3.8 Serum total GLP-1 concentration.....	85
Figure 4.1 body fat/ body weight in offspring	96
Figure 4.2 Fasting glucose insulin concentration in offspring.....	97
Figure 4.3 Pancreatic insulin content and beta cell mass in offspring.....	98
Figure 4.4 Cecal content pH and content weight in offspring	99
Figure 4.5 Growth curves of pups.....	100

ABBREVIATIONS

FBG	Fasting blood glucose
FBI	Fasting blood insulin
GLP-1	Glucagon-like peptide-1
GDM	Gestational Diabetes Mellitus
GK	Goto-Kakizaki
HOMA	Homeostatic Model Assessment
RS	Resistant starch
SCFA	Short chain fatty acids
UCP-1	Uncoupling protein 1

ABSTRACT

Treatment for maternal hyperglycemia is limited on account of safety concerns for the fetus. Our previous work has shown that inclusion of resistant starch in the diet decreases body fat accumulation in rodents, increases GLP-1 at both the gene expression level and plasma levels, and improves glucose tolerance in STZ-induced diabetic mice. However, studies concerning dietary resistant starch and maternal hyperglycemia are scarce. In this project, we examined the effects of dietary resistant starch in pregnant Goto-Kakizaki (GK) rats to improve glycemic control. We hypothesized that 1) dietary resistant starch could improve maternal glycemic control in a type 2 diabetes model –GK rat; 2) the favorable changes in dams would benefit offspring in terms of glucose metabolism.

Two animal experiments were conducted to test the hypotheses. In study 1, the female GK rats were randomly grouped to receive an energy control diet or resistant starch diet. The aged matched female Wistar rats were fed an energy control diet, serving as glycemia control. After 10 weeks on the assigned diets, all the female rats were mated with male Wistar rats and became pregnant. Fasting glucose concentration and fasting insulin concentration were measured on the 16th gestation day. In study 2, the offspring from different dams were fed on a chow diet until they reached 8 weeks old. At the end of the studies, body fat, glucagon-like peptide -1 (GLP-1), pancreatic insulin content, cecum pH, cecal short chain fatty acids levels, cecal butyrate producing bacterial profiles and β cell mass were measured.

Resistant starch fed GK rats had decreased body fat, improved insulin sensitivity (HOMA-IR), increased cecal short chain fatty acids and butyrate producing bacterial levels, and elevated plasma GLP-1. Also, GK rats on RS diet showed higher

beta cell mass compared with EC fed GK rats. Body weight and food intake were not changed by resistant starch. Offspring born to RS fed dams had lower fasting blood glucose and increased pancreatic insulin content. The feeding of RS to pregnant GK rats did not show negative impact on pup's growth and fetus survival rate. The conclusions are that dietary resistant starch was able to improve maternal hyperglycemia control in pregnant GK rats and decreased fasting glucose of their offspring without negative influences on growth and fetus survival rate.

CHAPTER 1

INTRODUCTION

The worldwide prevalence of diabetes has risen dramatically during the past two decades, especially in the United States. The 2007 national diabetes fact sheet (CDC, 2007) showed that 7.8% of the total population has diabetes, which is a 0.8% increase in two years (7.0% in 2005). With the incidence of diabetes hitting an all-time high, maternal hyperglycemia is becoming a health threat to pregnant women. Among 23.6 million people who suffer from diabetes at all ages, 23.5 million are age 20 and older, 11.5 million are women, a high portion of which are young patients whose ages fall in the range of 20 to 39, which happens to be the period of child bearing. In 2007, 281,000 new cases of diabetes were diagnosed in people aged from 20 to 39 years old (CDC, 2007). Maternal hyperglycemia not only brings negative impacts to pregnant women, but also an independent risk factor for negative fetus/infant's health conditions. Currently limited treatments are available for the safety concerns of the fetus. Therefore, it is important and urgent to find effective interventions to maternal hyperglycemia.

Resistant starches are non-digestible fermentable dietary fibers that resist digestion in the small intestine, but are fermented in the large intestine. It has been shown that adding resistant starch to diets produces several health benefits, including lower body fat storage (Shen et al, 2009; Keenan et al, 2006), and decreasing plasma glucose in rodents (Zhou et al, 2008). However, the knowledge on dietary resistant starch and maternal hyperglycemia is incomplete.

In addition to the conventional effects as dietary fiber, such as diluting the energy density of the diet and causing discomfort in the gut, resistant starch fed animals were also found to have significantly higher levels of glucagon-like peptide -1 (GLP-1)

(Keenan, et al. 2006; Zhou et al. 2006). The action of GLP-1, as a potent incretin, includes stimulating proinsulin gene expression (Drucker et al, 1987), inhibiting glucagon secretion (Nauck et al, 2002), mediating glucose-dependent insulin secretion via their receptors expressed on beta cells (Drucker, 2006), inhibiting gastric acid secretion and delaying gastric emptying(Baggio et al, 2004), as well as promoting an increase in pancreatic β -cell mass through enhancing beta cell proliferation and inhibiting apoptosis(Stoffers et al, 2003;Wang et al, 2002). GLP-1 was shown to be able to delay the onset of diabetes and improve pancreatic insulin content and total beta-cell mass in GK rats when applied postnatally for 5 days (Tourrel et al, 2002). GLP-1 also was reported to reduce apoptosis in human islets (Farilla et al, 2003). A GLP-1 receptor agonist demonstrated similar effects. Exendin-4 not only improved glucose tolerance in diabetic rats via expansion of beta cell volume (Xu et al, 1999), but also prevented the development of diabetes in rats exposed to intrauterine growth retardation (Stoffers et al, 2003).

The GK rat is a non-obese, polygenic model of type 2 diabetes derived from Wistar rats by selective breeding for slightly impaired glucose tolerance (Goto et al, 1975). This type of rat is characterized by moderate hyperglucemia, hypoinsulinaemia, normolipidaemia and impaired glucose tolerance which is thought to be the results primarily of reduced beta cell mass and a defective insulin response to glucose (Portha, 2005; Movassat et al, 1997; Movassat et al, 2007; Movassat et al, 1995). This model provides great opportunity to investigate the impact of dietary resistant starch on pancreatic beta cells.

Over the years, there has been a growing body of evidence indicating that intrauterine exposure to a hyperglycemic environment increased the risk of diabetes and

obesity for offspring later in their life in addition to any genetic transmission (Pettitt et al, 1999; Waterland et al, 1999; Boney et al, 2005). A study also revealed that glycaemic control in GDM pregnancies was an effective way to prevent impaired glucose tolerance in childhood. Even minimal intervention made a difference (Malcolm et al, 2006).

The current studies have been conducted to address the following questions: what would be the effects of dietary resistant starch on glycemic control in the pregnant GK rat? If there is improvement this should occur by improving insulin sensitivity through reducing body fat or through the involvement of GLP-1 on pancreatic beta cell functioning or both? Will the intervention influence their offspring? Will the possible discomfort in gastrointestinal tract due to high levels of fermentation induced by dietary resistant starch exert negative impact on gestation and the pup's growth? The hypotheses for this work are that 1) dietary resistant starch will improve maternal glycemic control in the GK rat— type 2 diabetes model and the elevation of GLP-1 might be an important factor involved; and 2) the favorable changes in the dams will benefit the offspring in terms of glucose metabolism.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Resistant Starch

2.1.1 General Introduction

Resistant Starches are dietary carbohydrates that resist digestion in the small intestine and reach the large intestine where they are fermented by bacteria to produce short chain fatty acids. Dietary resistant starches have been identified as possessing a variety of health benefits, which include decreasing plasma cholesterol and triglycerides, increasing satiety, producing anticancer effects, improving glucose tolerance, and consistently reducing body fat both in rodents and humans. (Zhou et al, 2008; Keenan et al, 2006; Brown 2004; Robertson et al, 2003; Shen et al, 2009).

However, the levels of resistant starch consumption have been progressively decreasing due to modern milling and food processing methods, especially in developed countries. Data show that in medieval Europe, the average intake of resistant starch was as high as 50-100 g/day (Birket 1997). And currently the estimated number for consumption of RS in developing countries is 30-40 g/day (Baghurst et al, 2001), whereas only 3-8 g daily are consumed by people in developed countries (Baghurst et al, 2001; Brighenti et al, 1998; Dyssler et al, 1994).

The term resistant starch (RS), was first coined in 1982 (Ritter et al, 1989). RS is divided into four subcategories: RS1, RS2, RS3, and RS4. RS1 represents starch in whole grains that are in a physically inaccessible form. RS2 is a type of starch, such as ungelatinized starch, which is tightly packed in a radial pattern in starch granules and resists digestion. The high amylose cornstarch used in the current study is an example of a RS2. RS3 is the type of starch that is most resistant to digestion. The starch fitting in

this category is mainly retrograded amylose formed in the process of cooling the gelatinized starch and can escape the digestion of pancreatic amylase almost totally. RS4 includes structurally modified starch by chemical treatment linking amylose strands. In our study, the starch used is composed of 60% amylose and 56% RS.

Recently dietary fermentable fibers have shown potential in the anti-diabetes field as a natural agent (Robertson et al, 2005; Park et al, 2004; Zhang et al, 2007). Our previous works show that dietary resistant starch possesses favorable impacts on gut hormone profiles, including promoting GLP-1 release consistently, a potent anti-diabetic incretin (Zhou et al, 2006). Also we demonstrated body fat was consistently lower in resistant starch fed animals compared to control animals fed the same energy density diet in rodents (Keenan et al, 2006; Shen et al, 2009). Additionally, we explored the effect of RS on glucose metabolism and found it lowers fasting glucose, fasting insulin and improves glucose intolerance (Zhou et al, 2008). Thus, resistant starch might be an alternative agent in diabetes treatment.

2.1.2 Resistant Starch and Glycemic Control

2.1.2.1 Animal Studies

- Feeding Resistant Starch Decreases Body Fat Accumulation in Rodents

Several studies demonstrated that rodents fed resistant starch had a significantly lower body fat (Shen et al, 2009; Keenan et al, 2006; Zhou et al, 2009). The RS induced body fat decrease is greater as higher levels of RS are in the diet and the longer the time of consumption. This has been shown in two different strains of rats (Wistar unpublished data; SD:Keenan et al, 2006) and in C57BL/6J mice (Zhou et al, 2009).

- Dietary Resistant Starch Lowers Fasting glucose, Fasting insulin and Improves Glucose Tolerance

C57BL/6J Mice fed RS had a significantly lower fasting glucose and insulin at 6

weeks (our unpublished data) .Diabetic mice induced by streptozotocin (STZ) were fed RS or control diet for 14days. Compared to the STZ injected mice fed control diet, the blood glucose levels and the area under the curve (AUC) during the OGTT significantly decreased ($P<0.05$) in STZ induced diabetic mice on the RS diet (Zhou et al, 2008). A study with 12 weeks of feeding dietary resistant starch to male GK rats inhibited glucose dependant insulintropic polypeptide (GIP) mRNA expression in the small intestine, improved fasting glucose level, but there was no significant change of GIP in the circulation (Shimada et al, 2008). Dietary RS also showed a positive effect on glycemic control in diet induced obese rats in a short interventional duration of 4 weeks. Aziz et al found that a high amylose starch diet, compared with an amylopectin rich diet, led to reduced fat mass, less incremental AUC of OGTT, decreased fasting serum glucose, insulin, leptin, a higher insulin sensitivity index (QUICKI), and an elevated mRNA expression of UCP-1(Aziz et al, 2009)

- Dietary Resistant Starch Increases Circulating Gut Hormones

Elevated plasma GLP-1 was observed in both normal SD and diet induced obese rats fed resistant starch (Shen et al, 2009; Aziz et al, 2009). And the GLP-1 increase is consistent over a 24 hour period (Zhou et al, 2008). Gene exxpression data from our group also verified that dietary RS dramatically up-regulated the expression of the GLP-1 gene in rat cecal cells compared to rats consuming an energy control diet (Zhou et al, 2006).

2.1.2.2 Human Studies

- Effect of RS on Glucose Metabolism

- **Long Term Studies**

Several human studies have been published regarding the effect of RS on

glucose metabolism. Among these studies different participants were recruited, however, the beneficial effects on metabolism were consistent and promising.

Early in 1995 de Roos et al found RS3 decreased insulin secretion while supplementation of RS2 resulted in lower appetite scores. In their study, 24 healthy males were supplemented with glucose, RS2 and RS3 for one week respectively and 48- hours of urine was collected to determine C-peptide for the last 2 days of each week. Satiety and food intake were measured using visualized analogue scale and a 24 hour recall method (de Roos et al, 1995).

In 2005, Robertson et al conducted a single-blind, crossover dietary intervention study in 10 healthy subjects that were given 30 g resistant starch per day for four weeks (Robertson et al, 2005). The resistant starch was in a form of Hi-Maize 260 at 50 g/d (30 g type 2 RS and 20 g rapidly digestible starch), and the placebo was 20 grams of Amioca starch which consisted of only rapidly digestible starch. The two starches were added to participants' habitual diet, separated by a 4-wk washout period. After RS treatment, insulin sensitivity became higher compared with placebo treatment when assessed by euglycemic-hyperinsulinemic clamp ($p=0.03$), and when using a meal tolerance test, it was 33% higher ($p=0.05$), skeletal muscle glucose clearance was also significantly higher ($p=0.027$). With resistant starch supplementation, subcutaneous abdominal adipose tissue nonesterified fatty acid (NEFA; $P = 0.02$) and glycerol ($P = 0.05$) release were lower than with placebo. However, plasma GLP-1 and leptin concentrations were not significantly changed. Systemic acetate and propionate concentrations were significantly higher (by repeated measure) as well as fasting plasma ghrelin with RS intervention.

A similar study in overweight subjects was published by Park et al in 2004 (Park et al, 2004). It was a double –blind randomized study. The investigators recruited 25

overweight participants (defined by over 120% of their ideal weight), 12 in treatment and 13 in control, feeding them either amylose 24g each day for 21 days or the same amount of regular corn starch with regular meals. At the end of the study, participants supplemented with RS had lower total serum cholesterol, LDL-cholesterol, and fasting serum glucose concentration ($p < 0.05$).

Robertson and her coworkers did another randomized, crossover study in overweight individuals with metabolic syndrome in 2009 (Robertson et al, 2009). They recruited 10 overweight individuals with metabolic syndrome and fed them 40 grams of resistant starch from Hi-maize per day for 4 weeks, and control regular starch for another 4 week, set apart by a washout period. By using an arteriovenous difference method, they found the glucose clearance in forearm skeletal muscle was increased by 68% with resistant starch supplementation. Moreover, the subjects who consumed RS had improved hepatic insulin sensitivity and peripheral insulin sensitivity as well. These parameters were all measured by the hyperinsulinemic-euglycemic insulin clamp and the indirect meal tolerance test. The results of the latter also showed consumption of RS can reduce fasting insulin levels, postprandial insulin responses to the meal, and fasting free fatty acids concentrations.

Another study was done in 20 metabolic syndrome subjects with 40 grams a day of resistant starch or placebo supplement (0 g/day) for 12 weeks by the same group (Johnston et al, 2010). Resistant starch consumption improved insulin sensitivity measured by euglycaemic-hyperinsulinaemic clamp, compared with the placebo group ($P = 0.023$). However, this change was not accompanied by the alteration of fat storage in muscle, liver or visceral depots and inflammatory markers.

A randomized, crossover study in type 2 DM patients was conducted by Zhang et al in 2007(Zhang et al, 2007). Forty type 2 diabetics were randomly assigned into group A and group B. At stage I, group A received 30 g resistant starch from Hi-maize per day for four weeks and group B served as control. In stage II, the two groups switched onto the opposite treatments for the same period of time. With consumption of resistant starch, fasting blood glucose, post prandial blood glucose, total cholesterol, and triglycerides were significantly lower in the intervention group ($P < 0.05$). The insulin sensitivity index calculated with fasting glucose and insulin concentrations was higher in the intervention group than in the control group ($P < 0.05$) as indirectly measured by a meal tolerance test.

➤ **Short Term Studies**

Robertson and her coworkers evaluated the short term effect of dietary resistant starch on glucose metabolism in healthy subjects in 2003 (Robertson et al, 2003). In this single-blind, crossover dietary intervention study, 10 healthy subjects were given a basal diet either supplemented with 100 gram Himaize (consisting of 60 g RS and 40 g rapidly digestible starch) or with 40 g of rapidly digestible starch for 24 hours. The starch was mixed with jelly and served. Insulin sensitivity was assessed by meal tolerance test in combination with the minimal model index. Prior RS consumption led to decreased glucose excursion during the 5 hour MTT test as well as the insulin concentration. Systemic insulin sensitivity was improved significantly ($p=0.028$). No significant effects of dietary RS on plasma GLP-1, short-chain fatty acids and plasma triacylglycerol were observed.

A double-blind, placebo-controlled, crossover dietary intervention study was performed in participants with fasting blood glucose between 100mg/dl and 140mg/dl

(Yamada et al, 2005). Twenty subjects ingested bread either containing 6 gram of RS3 or no RS separated by 2 weeks. Blood was drawn from subjects for blood glucose and insulin measurement prior to ingestion and 0.5, 1, 1.5 and 2 hours following ingestion. According to their fasting blood glucose, they were divided into a borderline group (blood glucose ≥ 111 mg/dl) and a normal group. Postprandial blood glucose increase was reduced significantly at 1 hour and 1.5 hour after ingestion of RS bread compared with placebo. So was the insulin response. However, these changes occurred in the borderline group and not in subjects from the normal group.

Achour and coworkers recruited 8 healthy subjects and gave them two test meals at 0800 and 2200 respectively in a 27 hour period (Achour et al, 1997). The test meal consisted of either 50g pre-gelatinized corn starch or 50g retrograded corn starch. As a placebo-controlled, crossover dietary intervention study, the subjects had two test periods, with one week interval between. The diet composition for the test meal and placebo meal was the same except for the starch type. However, they didn't count the difference in metabolized energy between these two starches. An 8-hour period right after ingestion of the first meal was designated as the absorptive period. The postabsorptive period was defined as a 3 hour duration 10 hours after consumption of the second test meal. Fasting blood glucose, insulin, glycerol, fatty acids, acetate and breath hydrogen, breath methane were measured.

Blood glucose and insulin were lower with ingestion of retrograded starch in the absorptive stage. No other significant differences were observed in blood acetate, free fatty acids, respiratory quotient, breath hydrogen and breath methane. Blood glycerol was lower after ingestion of pre-gelatinized starch. In the postabsorptive period blood glucose and insulin were not different between the two meals. However, other measurements

were significantly higher or lower (glycerol) with retrograded corn starch except for the free fatty acids concentration. They reported that fermentation occurred for 24 hours after consumption as indicated by elevation of breath hydrogen and blood acetate. The result was consistent with Liljeberg's observation in 1999 that a 4 –hour digestion was not long enough to observe fermentation of RS (Liljeberg et al, 1999).

- Dietary Resistant Starch and Maternal Hyperglycemia

So far there is no study available that has investigated the effect of RS on maternal hyperglycemia. However, there is evidence that dietary fiber intake was strongly and inversely associated with gestational diabetes risk (n=13,110; 8 year follow-up) (Zhang et al, 2006). A low glycemic index diet has also been shown to significantly decrease birth size, birthweight, and ponderal index of infants born to healthy mothers (Moses et al, 2006). Table 2.1 summaries published human studies of glycemic index and Maternal Hyperglycemia.

Table 2.1 Summary of published human studies of glycemic index and maternal hyperglycemia

Purpose	Participant #	Diet	Results	Reference
LOW GI vs high(conventional high-fiber) If reduce the need of insulin	63 GDM		(9/31) 29% vs 59%(19/32) 9/19 stop insulin after changing to LGI diet	Moses et al, 2009
52% CHO composed of low and moderate glycemic index in controlling GDM condition	31 GDM 76% OB, 24% OW	52% carbohydrates , 30% of fat, 18% of proteins 4 months	GLU :146 +/- 37 vs 90 +/- 5 mg/dL Hb1 A c: 7.1 +/- 1.2 and 5.0 +/- 0.7	Monroy et al, 2008
The association between total dietary fiber consumptions and GDM	758 GDM	prospective cohort study in the Nurses' Health Study II	10-g/day ↑~26% ↓ in risk; 5-g/day ↑ in cereal or fruit fiber ~ 23% (9-36) or 26% (5-42) ↓	Zhang et al, 2006

(Table 2.1 con'd)

Improving glucose tolerance, insulin sensitivity and lipids file by changing the glycaemic index (GI) and dietary fibre (DF) content of their bread	7 IGT With previous GDM	Crossover study Low GI/high DF vs high GI/low DF for 3 week	Improved insulin economy based on the lowered insulin responses to the intravenous glucose challenge (35%) No changes in fasting levels of glucose, insulin, HDL-cholesterol or TG.	Ostman et al, 2006
influence of different glycemic index diet on infant birth weight, fetal growth, and biomarkers of carbohydrate metabolism	1,082 health pregnant women	Prospective cohort study in The Camden Study	glycemic index was positively related to maternal Hb1Ac ($\beta=0.004$) and plasma glucose ($\beta=0.163$), $p<0.05$	Scholl et al, 2004
Trial 1: high-fiber diets compared with normal pregnancy diets Trial 2&3: LGI diet compared with high glycaemic index diet	Trial 1:25 Trial 2&3: 87	Meta-analysis	Trial1: no difference Trial2&3: fewer large for gestational age infants; lower ponderal indexes: lower maternal fasting glucose levels	Review (Tieu et al, 2008)
Whether higher dietary fiber intake (water soluble and insoluble) is associated with lower insulin requirements and better glycemic control	141 pregnant with 1-DM	Observational study Total fiber	1. Higer fiber intake(20.5g/d)vs lower fiber intake(8.1g/d) required 16%~18% less insulin. 2. fiber intake was not associated with FBS and Hb1Ac	Kalkwarf et al, 2001

2.1.2.3 Possible Mechanisms

The mechanism involved in improving glycemic control by resistant starch is not completely understood. As a part of the diet, RS potentially has three major effects (Keenan et al, 2006): metabolizable energy dilution, a bulking effect and fermentation to

produce short-chain fatty acids and increase PYY and GLP-1 through nutrient-gene interactions. Resistant starch dilutes the energy density of the diet, which may contribute to better glycemic control by reducing caloric intake. However, in our previous studies, we balanced the energy density in the two diets, RS and EC, to exclude the effects of energy dilution, and still obtained a similar outcome. Another assumption is that fermentation of resistant starch causes discomfort in the gut, which leads to decreased food intake and less glycemic load after meals. Nevertheless, resistant starch fed animals ate the same or more food than controls (Keenan et al, 2006; Zhou et al, 2008), which counters the previous assumption. The data from our lab showed dietary resistant starch reduced body fat, increased fatty acid oxidation and promoted energy expenditure in rodents (Zhou et al, 2008). However, failure of fermentation wiped out the effects (Zhou et al, 2009). We also demonstrated gene expression and plasma levels of GLP-1 and PYY were elevated in the large intestine where fermentation occurs (Zhou et al, 2006). Thus, the fermentation of resistant starch in the large intestine by gut microflora, which produces the increase of short chain fatty acids and consequent increase of gut hormones , might be considered as a major factor for RS improving glucose metabolism.

- Fermentation in the Gut

The process of fermentation of RS in the large intestine involves several groups of bacteria. When RS reaches the hind gut, bacteria such as *Bacteroides thetaiotaomicron* (*Bacteroides* spp), *Bifidobacterium longum* (*Bifidobacterium* spp) and some *Lactobacillus* spp (Bird et al, 2000; Xu et al, 2003a; Louis et al, 2007b) will adhere to the surface of starch molecules and begin to convert RS into their fermentation products. The end products of *Bifidobacterium* spp and *Lactobacillus* spp in fermenting RS are lactate and acetate, whereas acetate, propionate and succinate are produced by *Bacteroides* spp

(Duncan et al, 2002; Louis et al, 2007a). However, fermentation does not end here. These products are intermediate metabolites that will be further converted to butyrate by other bacterial species (Duncan et al, 2004a; Duncan et al, 2004b).

The majority of the butyrate producing bacteria belongs to Clostridium cluster IV/*C. leptum* group and Clostridium cluster XIV /*Clostridium coccoides*–Eubacterium rectale group (Louis et al, 2007a; Sato et al, 2008). Some of them are capable of utilizing acetate to produce butyrate, such as Coprococcus spp. and Roseburia spp. of Clostridium cluster XIV and Faecalibacterium prausnitzii of Clostridium cluster IV (Duncan et al, 2002; Cani et al, 2007a). Others like Eubacterium hallii and Anaerostipes caccae are able to catalyze both the D and L isomers of lactic acid to butyrate (Duncan et al., 2004b), whereas Eubacterium limosum can convert lactate into acetate and butyrate with the presence of Bifidobacterium longum (Sato et al, 2008).

- Increase of Glucagon-Like Peptide -1

It is reported that resistant starch fed animals have significantly a higher level of glucagon-like peptide -1 (GLP-1) (Keenan, et al. 2006; Zhou et al. 2006). GLP-1 is a satiety peptide yielded from the pre-proglucagon gene product in the L enteroendocrine cells of the distal intestine (Badman et al, 2005)

GLP-1 has several forms in the circulation. The inactive forms, GLP-1₁₋₃₆ and GLP-1₁₋₃₇, are cleaved from preproglucagon, depending on whether the C-terminal glycine is present. Further N-terminal truncation is required to produce the biologically active forms, GLP-1₇₋₃₆ and GLP-1₇₋₃₇ (Mojsov et al. 1986).

GLP-1 is released into the circulation in a biphasic manner in proportion to the calories ingested (Orskov et al. 1994). The early phase release seems to be mediated by a

neuroendocrine reflex, whereas the latter is a result of the presence of undigested nutrients present in the lumen of the ileum or large intestine with L-cells.

The action of GLP-1, as a potent incretin, including mediating glucose-dependent insulin secretion via their receptors expressed on beta cells (Drucker, 2006), stimulating proinsulin gene expression (Drucker et al, 1987), inhibiting glucagon secretion (Nauck et al, 2002), inhibiting gastric acid secretion and delaying gastric emptying (Baggio et al, 2004), as well as promoting an increase in pancreatic β -cell mass through enhancing beta cell proliferation and inhibiting apoptosis (Stoffers et al, 2003; Wang et al, 2002).

GLP-1 was shown to be able to delay the onset and improve the severity of diabetes in GK rats when given postnatally. GK rats with daily injections of glucagon-like peptide-1 (400 microg x kg⁻¹ x day⁻¹) from day 2 to day 6 exhibited higher pancreatic insulin content and total beta-cell mass than control rats. There were long term effects observed in the same study: lower basal plasma glucose and slightly increased glucose stimulated insulin secretion were found two months later in treated GK rats (Tourrel et al, 2002). GLP-1 also was reported to reduce apoptosis in human islets (Farilla et al, 2003). A GLP-1 receptor agonist demonstrated similar effects. Exendin-4 not only improved glucose tolerance in diabetic rats via expansion of beta cell volume (Xu et al, 1999), but also prevented the development of diabetes in rats exposed to intrauterine growth retardation (Stoffers et al, 2003).

- Short Chain Fatty Acids (SCFA)

The main products of fermentation of dietary fibers in the mammalian gut are SCFA including acetate, propionate, and butyrate (Morita et al, 1999). The typical ratios of acetate to propionate to butyrate in feces are 3:1:1 (Duncan et al, 2002). Normally,

acetate is circulating in the bloodstream in a concentration around 100–150 μM , 4–5 μM for propionate, and 1–3 μM for butyrate (Wolever et al, 1997).

A supplement of sodium butyrate facilitated fatty oxidation and increased energy expenditure in mice; it protected mice from insulin resistance and hyperglycemia in diet induced obesity (Gao et al, 2009). As one of the products from fermentation, it is speculated that butyrate might be a key factor to mediate the RS signal to regulate glucose metabolism. RS probably increases butyrate producing bacteria in the lower gut. The elevated butyrate appears to stimulate GLP-1 expression and release from L-enterocytes through HDAC inhibition (Zhou et al, 2006). Increased GLP-1 signals to the hypothalamus to upregulate hypothalamic POMC (Shen et al, 2009) which is an important neuropeptide in promoting energy expenditure (Santini et al, 2009). In addition, GLP-1 was reported to induce cAMP in hepatocytes (Ding et al, 2006), so it may upregulate peroxisome proliferator-activated receptor (PPAR)- γ coactivator (PGC)-1 α activity through the PKA-CREB pathway (Puigserver et al, 2003; Lin et al, 2005). PGC-1 α is a transcription coactivator, which controls energy metabolism by targeting several transcription factors of genes involved in fatty acid oxidation and mitochondrial function which are essential in glucose metabolism (Lin et al, 2005).

After being transferred to blood stream, butyrate fed in the diet may directly activate PGC-1 α and uncoupling protein (UCP)-1 in brown fat, PGC-1 α and adenosine monophosphate-activated protein kinase (AMPK) in muscle and liver cells (Gao et al, 2009). In the muscle, PGC-1 α increases fatty acid metabolism (Lin et al, 2002). In brown fat, PGC-1 α enhances adaptive thermogenesis through upregulation of UCP-1 expression (Puigserver et al, 1998). UCP-1 diverts energy from ATP synthesis to thermogenesis to increase energy expenditure. It has been shown that AMPK and p38 is responsible for

upregulating PGC-1 α activity in the post-translational phase (Knutti et al, 2001; Jager et al, 2007). Butyrate was demonstrated to increase PGC-1 α expression through both ways (Gao et al, 2009). In addition, AMPK is an important regulatory sensor promoting fatty acid oxidation and glucose metabolism (Gruzman et al, 2009).

As a histone deacetylase inhibitor, butyrate may also activate PPAR α in peripheral tissues. In vitro, butyrate enhanced the transcriptional activity of PPAR α in a dosage dependent manner. PPAR α plays a critical role in activation of mitochondrial β -oxidation of fatty acids through regulation of genes encoding key enzymes such as carnitine palmitoyltransferase I, acetyl-CoA synthase and so on (Komatsu et al, 2010). PPAR α interacts with PGC-1 α in metabolically active tissues including liver, skeletal muscle, and brown fat and induces the expression of PPAR α target genes (Vega et al, 2002). In liver and muscle, PGC-1 α facilitates PPAR α in the regulation of fatty acid oxidation. In liver, they cooperatively control glucose production (Berger et al, 2002; Francis et al, 2003). However, further study is needed to identify whether PPAR α and PGC-1 are required for butyrate induced energy expenditure.

2.2 Maternal Hyperglycemia

2.2.1 Current Problems

The worldwide prevalence of diabetes has risen dramatically during the past two decades, especially in the United States. The 2007 national diabetes fact sheet (CDC, 2007) showed that 7.8% of the total population has diabetes, which is a 0.8% increase in two years (7.0% in 2005). Among 23.6 million people who suffer from diabetes at all ages, 23.5 million are age 20 and older, 11.5 million are women, quite a portion of which are young patients whose ages fall in the range of 20 to 39, which happen to be the period of child bearing. In 2007, 281,000 new cases of diabetes were diagnosed in people aged

from 20 to 39 years old (CDC, 2007). In context of the current diabetes prevalence, maternal hyperglycemia is becoming a health threat to pregnant women worldwide, which consists of diabetic pregnant women (majority is type 2 diabetes) and those diagnosed with Gestational Diabetes Mellitus (GDM). Maternal hyperglycemia not only brings negative impacts to pregnant women, but also is an independent risk factor for fetus/infant's health conditions. Gestational diabetes mellitus, a special type of diabetes in pregnancy, is further elucidated as follows.

2.2.1.1 Gestational Diabetes Mellitus

GDM is defined as the onset of glucose intolerance in a pregnant woman who has not been diagnosed with diabetes before pregnancy. As a result of the increase in prevalence, the severe adverse outcomes it brings to both mothers and their offspring, and the limited treatment variety, GDM becomes a leading threatening factor to the health of young women and their babies.

- Increase of GDM incidence

The incidence of GDM is growing. It has doubled over the last few years and the trend continues. In the United States, the rates per 1000 women have increased more than 3.5-fold among those aged 15–24 and 4 –fold in the age range 25–34 (Baraban et al, 2008). Data from the American Diabetes Association indicate gestational diabetes occurs in about 4% of all pregnant women, with 135,000 new cases in the USA each year (ADA.org). In 2006, the National Institute of Child Health and Human Development estimated that GDM affects nearly 7 percent of all pregnancies (NIH.gov). The prevalence of GDM has similar features to the obesity epidemic. 1. The incidence is higher in urban versus semi-urban and rural areas in a developing country. Seshiah et al conducted a study with a total of 41,513,960 participants in South India. They found the

prevalence was 17.8% women in urban, 13.8% in semi-urban, and 9.9% in rural areas (Seshiah et al.2008). 2. In the same ethnic groups, people who live in s developing country have a lower prevalence than those who immigrated to western countries (2.7% vs 7.3%) (Yang et al, 2002).

- Adverse Outcomes

GDM has severe effects on both mothers and their offspring. Mothers with GDM have an increased lifetime risk of developing type 2 diabetes mellitus (DM). According to data reported by CDC, 5 to 10 percent of women with GDM will be diagnosed as type 2 DM right after pregnancy. Even for those women who return to a normal glucose metabolism, they still have a 20 to 50 percent chance of developing diabetes in the next 5 to 10 years. (<http://www.cdc.gov/diabetes/pubs/interim/background>)

As for the infant, GDM has a more complicated impact. In the short-term, infants born to parents with GDM have a higher chance of having macrosomia and consequenced dystocia, developing respiratory distress syndrome and jaundice, stillbirth, and dying in infancy. For the long-term, GDM is a significant risk factor for development of obesity and abnormal glucose metabolism in their late life (Metzger, 2007).

It was shown that infants of GDM mothers had significantly greater skinfold measures as well as fat mass, even when they are not macrosomic, compared to those of mothers with normal glucose tolerance (Catalano et al, 2003). The increase of fetal adiposity is thought to be a predictor for obesity in early childhood (Simmons, 2008).

The study of 552 Pima Indian offspring of GDM patients aged 5-24 yr showed that a 1 mM higher maternal 2-h glucose level resulted in a higher occurrence of diabetes in the offspring (odds ratio = 162) (Pettitt et al, 1991). The observation was not only limited to Pima Indians, but also occurs in the general population. Children who are exposed to

an intrauterine environment of a high glucose concentration are at increased risk of developing metabolic syndrome: 50% of the GDM group confirmed more than 2 components of metabolic syndrome, which was significantly higher than the control group, 18% (Boney et al, 2005). One study revealed that glycaemic control in GDM pregnancies is an effective way to prevent impaired glucose tolerance in childhood. Even minimal intervention will make a difference (Malcolm et al, 2006).

- Limited treatment variety

So far, treatment for GDM is limited only to nutrition intervention and insulin. Nutrition therapy is the primary treatment for about 70-90% of women with gestational diabetes mellitus (Gunderson, 2004). It has been recognized as the cornerstone of therapy for GDM at worldwide conference (Metzger, 1998). The goal of nutrition therapy is to achieve a balance between adequate nutritional needs and optimal glycemic control. Although caloric restriction of 35-40 percent of total calories is effective for treatment for GDM (Major et al, 1998), ADA suggests that it should be done with great caution because of ketonemia and ketonuria, which can harm the fetus (American Diabetes Association, 2002). Moreover, compared to numerous treatments available for Diabetes, insulin is the only currently FDA approved medication for GDM. Therefore, new treatments for GDM are necessary.

2.2.2 Mechanisms of Insulin Resistance in Maternal Hyperglycemia

Insulin resistance is thought to be the potential pathophysiology for GDM (Buchanan et al, 2007). Progressive insulin resistance is present during normal pregnancy due to elevated placental hormones and increased maternal adiposity (Barbour et al, 2007). It usually begins near mid-pregnancy and reaches to the levels approaching what seen in type 2 diabetes in the third trimester. Therefore, pregnancy is considered as

a "diabetogenic state "(Zavalza-Gómez AB et al, 2008). There is molecular evidence that with GDM there is greater insulin resistance than with normal pregnancy (table 2.2). So GDM occurs when people who already have a chronic insulin resistance background undergo the “diabetogenic state.”

Table 2.2 Comparison of normal pregnancy and gestational diabetes in terms of insulin resistance

	GDM	Normal pregnancy	
Insulin stimulated Glucose transport Muscle ^(Friedman, 1999)	65% ^↓	40% *↓	Change of glut4 protein is undetectable
Adipose ^(okuno, 1995)	GLUT4 ↓↓	↓	
Tyrosine phosphorylation of IR ^{^ (Friedman, 1999)}	↓	—	
Activity of IR pretreated with insulin ^{(shao,2000a)*}	↓↓ Partially restored by alkaline phosphatase	↓ Restored to normal	
IR-1 protein* Muscle ^(Friedman, 1999)	↓↓	↓	
Adipose ^(Catalano,2002)	↓	No change	
Antepartum Postpartum	52%↓ Back to normal	No change No change	
Serine phosphorylation of IRS-1 ^{^ (Barbour, 2006)}	62% ↑ S-IRS-1/Total IRS-1	—	
P85αmonomer ^(Catalano,2002)	↑*	↑*	
^ compare with normal pregnancy;	* Compare with non- pregnant obese women		

2.2.2.1 Pregnancy Induced Insulin Resistance

Insulin resistance identified in pregnancy returns to normal soon after pregnancy and so do the signaling changes in women with normal glucose tolerance. These findings suggest that pregnancy per se is capable of inducing insulin resistance. As for mechanism, several lines of evidence suggest that the physiological insulin resistance induced by pregnancy could be caused by post-receptor defects and appear to be multifactorial (Boyd et al, 2007). Normally, insulin binds to the α subunit of the insulin receptor, and catalyzes autophosphorylation of the β -subunit of the insulin receptor at tyrosine residues, then the activated receptor docks the insulin receptor substrate and tyrosine phosphorylates this protein, followed by recruiting of P85 of phosphatidylinositol 3-kinase (PI3), production of PIP3 and signaling for GLUT4 translocation (Aguirre et al, 2002).

However, in pregnant women, a significant decrease in insulin receptor tyrosine phosphorylation in muscle has been identified. In the light of evidence that the tyrosine phosphatase activity and receptor number are not changed (Friedman et al, 1999, Shao et al, 2000a), and the pretreating insulin receptor with alkaline phosphatase could restore insulin's ability to activate tyrosine phosphorylation of the insulin receptor (Shao et al, 2000b), it is suggested that increased serine phosphorylation of the insulin receptor and subsequently competitively downstream insulin signaling plays an important role. At the level of insulin receptor substrate-1 (IRS-1), strong evidence is also presented for activated IRS-1 serine phosphorylation (Qiao et al, 2002). IRS-1 serine phosphorylation is not only able to accelerate its degradation, but also dampen PI3 kinase activity by inhibiting recruitment of P85 subunit. Possible mechanisms include 1. Excessive nutrients such as amino acid and glucose during pregnancy stimulate p70 S6K expression, which increases serine phosphorylation (Shah et al, 2006); 2. TNF- α augmented by both

placenta and expanded adipose tissue during pregnancy upregulates JNK, and this can catalyze phosphorylation on serine residues (Aguirre et al, 2000); 3. Both TNF- α and growth hormone can suppress expression of peroxisome proliferator activated receptor which is a transcription factor for adiponectin (Masternak et al, 2005, Qiao et al, 2005). Additionally, a decreased adiponectin level leads to increased activity of the mTOR pathway, which also contributes to serine phosphorylation (Tzatsos et al, 2006).

In addition, the increased amount of the free p85 α subunit of PI3 kinase appears to be involved in the post receptor defects (Barbour et al, 2005). PI3 kinase has two subunits, the regulatory p85 α subunit and catalytic p110 subunit. IRS has to bind with p85 α - p110 heterodimer to activate this enzyme and produce PIP3. P85 α monomers block IRS docking the p85 α - p110 heterodimer (Biddinger et al, 2006). Placental growth hormone appears to be involved in the accretion of P85 α monomers (Barbour et al, 2002).

2.2.2.2 Role of Placenta in Insulin Resistance

The placenta is a complicated and crucial fetal organ for fetal growth. It is in contact with both maternal and fetal circulations through different surfaces. Therefore, it could be influenced by changes of regulatory factors present in the two circulations and as a feedback produces hormones and cytokines to act on mother and fetus (Desoye et al, 2007).

Normally, during gestation the placenta synthesizes and releases a variety of cytokines which contribute to the low grade systemic inflammation, and together with hormones secreted by the placenta as well, are necessary to induce maternal insulin resistance (Barbour et al, 2004). This physiological insulin resistance in late pregnancy is a critical adaptation designed to ensure the growing fetus has an adequate supply of nutrients by blunting maternal glucose uptake.

However, in a pregnancy complicated with diabetes mellitus, the abnormal maternal metabolic environment may stimulate the overexpression of cytokines and inflammatory- related genes in the placenta and this results in the increased circulating concentrations of inflammatory cytokines (Hauguel-de Mouzon et al, 2006). There are a high amount of insulin receptors expressed in the placenta. It has been suggested that, during the pregnancy, altered maternal insulin lead to abnormal synthesis and secretion of hormones and cytokines by the placenta by interacting with the syncytiotrophoblast, and this contributes to maternal insulin resistance (Desoye et al, 2007).

A variety of hormones secreted from the placenta and circulated in high concentrations have been shown to be able to influence the insulin sensitivity in peripheral tissues and induce insulin resistance during pregnancy; Estrogen, progesterone, human placental lactogen (hPL), and human placental growth hormone (hPGH) are reported to be involved in the process. (Barbour et al, 2004; Nagira et al, 2006; Kühl , 1998; Reis et al, 1997; Ryan et al, 1988).

In surgical postmenopausal monkeys, estrogen and medroxyprogesterone acetate treatment led to enlarged adipocytes and diminished insulin sensitivity (Shadoan et al, 2007). It was suggested that a post-receptor mechanism of activation of JNK mediated by the membrane estrogen receptor (ER) pathway and the subsequent serine phosphorylation of IRS1 probably contribute to estrogen induced insulin resistance in 3T3-L1 adipocytes (Nagira et al, 2006). Estradiol also decreased insulin sensitivity by down- regulating muscle glucose transport 4 (GLUT4) in vivo; when at high concentrations similar to that in pregnancy, it reduced GLUT4 expression by 30% at both mRNA and protein levels. Time- and dose-dependent responses were observed as well in the L-6 cell line. (Barros et al, 2008);

The knockout of the progesterone receptor was reported to promote beta-cell proliferation and improve insulin secretion in response to glucose injection in female mice which overall showed ameliorated glucose homeostasis (Picard et al, 2002). In vitro, progesterone was shown to inhibit glucose transport as well as reduce glycogen synthesis and glycolysis regardless of the presence of insulin in muscle (Leturque et al, 1989). The mechanism studies indicated that the inhibition was obtained by suppressing multiple steps of insulin signaling which included decrease of IRS1 expression and suppression of the PI3-kinase independent pathway of TC10 activation (Wada et al, 2010).

It has been postulated that human placental lactogen (hPL) is the major placental hormone which reprograms the occurrence of insulin resistance. Human placental lactogen (hPL) rises 30-fold during pregnancy (Brelje et al, 1993). As early as in 1967, hPL was indicated to induce peripheral insulin resistance in normal male subjects (Beck et al, 1967). Placental lactogen was considered one of the hormones that were related to the postreceptor defect of insulin signaling during pregnancy (Ryan et al, 1988). The opposite result occurred with ovine placental lactogen's failure to deteriorate glucose metabolism in skeletal muscle (Leturque, 1989). However, overall supportive evidence of hPL as the key factor in insulin resistance during pregnancy is sparse. On the contrary, more and more studies suggest the main effect of hPL is to induce the pregnancy-associated growth of pancreatic islets and insulin secretion (Yamashita et al, 2000; Handwerger et al, 2000; Brelje et al, 1993).

Human placental growth hormone (hPGH) is another placental hormone related to the occurrence of pregnancy –induced insulin resistance, and is so far the most likely hormonal candidate which mediates the insulin resistance in pregnancy (Barbour et al, 2004). Human placental growth hormone rises up to eightfold during pregnancy and is

free from regulation by growth hormone release hormone (GHRH) (Handwerger et al, 2000). Severe peripheral insulin resistance was observed in transgenic mice overexpressing placental growth hormone (Barbour et al, 2002). Further research indicated this insulin resistance was due to the increased expression of one of the subunits of PI 3-kinase, p85 monomer, and subsequent disruption of PI 3-kinase activation in skeletal muscle. (Barbour et al, 2004). PI 3-kinase activation is a critical step in the insulin signaling pathway whose product is required for propagating signals for GLUT4 translocation. Phosphorylated insulin receptor substrate 1 (IRS-1) must dock regulatory p85 α subunit and the catalytic p110 subunit as a heterodimer in order to activate the enzyme (Wymann et al, 1998). p85 α competes with the heterodimer to bind IRS-1 at a specific binding site and effectively prevents the access of the heterodimer to IRS-1 once it succeeds (Barbour et al, 2004). The p85 α heterozygous deletion mice (p85 α +/-) demonstrated p85 α is a key mediator in the placental growth hormone's induction of insulin resistance. Placental growth hormone caused overexpression of p85 α , diminished insulin stimulated PI 3-kinase activity and increased insulin resistance in wild type mice; whereas p85 α +/-mice were protected from its influence as evidenced by maintained global insulin sensitivity and PI 3-kinase activity ((Barbour et al, 2005).

In addition to hormones, the human placenta also synthesizes virtually all known cytokines which may contribute to the inflammation in the whole body (Telejko et al, 2010). Kirwan and colleagues investigated the correlation between insulin sensitivity and placental products that included tumor necrosis factor α (TNF- α), leptin, cortisol, human chorionic gonadotropin, es-tradiol, progesterone, human placental lactogen, and prolactin in pregnancy. They reported that in humans, among all variables they studied, only the circulating plasma TNF α produced by the placenta was inversely correlated highly with

in vivo insulin sensitivity, while hPGH was not included in this study (Kirwan et al, 2002). In obese pregnant women a similar association was observed between increased TNF- α in peripheral blood and insulin resistance (Challier et al, 2008). Infusion of TNF- α in rat led to increased global insulin resistance, and blunted glucose uptake when incubated with human skeletal muscle cells in culture (Frost et al, 2005).

Possible mechanisms were suggested. The first is that TNF- α is able to act as a serine threonine kinase to catalyze insulin receptor (IR) and IRS-1 serine phosphorylation which in turn inhibits their tyrosine phosphorylation (Barbour et al, 2006; Catalano et al, 2002). TNF - α activated JNK, which can phosphorylate IR and IRS-1 on serine residues (Rui et al, 2001). The second possible mechanism is that TNF- α has been shown to suppress PPAR γ expression in the 3T3-L1 cell line and inhibit adipocyte differentiation (Zhang et al, 1996). PPAR- γ is a transcription factor, expressed in adipose tissue, and is essential in regulation of insulin sensitivity, adipocyte differentiation, and lipid storage (Joosen et al, 2006; Zeghari et al, 2000; Schoonjans et al, 1996). One of its important target genes is adiponectin (Qiao et al, 2005). Adiponectin is viewed as an endogenous insulin-sensitizer due to its favorable effect on glucose homeostasis through its receptors in skeletal muscle and liver (Hara et al, 2005). Several studies indicated that circulating adiponectin level decreased in obesity and insulin resistance animal models (Arita et al, 1999). Epidemiology studies show an inverse correlation between plasma adiponectin concentrations and the degree of insulin resistance in obesity, type 2 diabetes, and GDM (Weyer et al, 2001; Worda et al, 2004; Cseh et al, 2004)).

Leptin is another placenta cytokine widely studied. Although adipose tissue secretes leptin as does the placenta during gestation, it is suggested that the major changes in leptin in pregnancy are attributed to placental leptin production (Bajoria, et al,

2002). Several studies reported leptin was correlated negatively with insulin sensitivity in normal pregnant women and gestational diabetics (McIntyre et al, 2010; Mastorakos et al et al, 2007). However, divergent findings about cytokines and insulin sensitivity are not scarce which may be due to variation in experiment design, methods adopted, and different subjects recruited. (Briana et al, 2009).

Further study is still needed to explore later steps in the insulin-signaling pathway and examine the complicated cytokine and hormone crosstalk between placenta and enlarged adipose tissue.

2.3 Beta Cell Dysfunction

Impaired beta cell function as well as tissues insulin resistance are two etiologic factors in type 2 diabetes, which counts for 90-95% of total diabetes. It was thought insulin resistance was the primary cause because it was more frequently reported preceding beta cell dysfunction in the pathogenesis of type 2 diabetes (Gerich et al, 1999). Besides, insulin resistance is highly correlated with both BMI and type 2 diabetes, therefore, it may link overweight with increasing glycemia (Balkau et al, 2002). It also appears to induce beta cell dysfunction through beta cell exhaustion (Leahy et al, 2005). However, Gerich and co-workers found impaired beta cell secretion were present in non-diabetic first degree relatives of type 2 diabetes patients (Gerich et al, 2003). Moreover, compared with controls, the first degree relatives exhibited reduced first and second phase insulin secretion but no difference in insulin resistance (Pimenta et al, 1995). Albeit there is still gap in our knowledge, it is well accepted that progressive beta cell dysfunction is a necessary condition in the occurrence of diabetes (Marchetti et al, 2008).

2.3.1 Reduced Beta Cell Mass

Normally there are about one million islets of Langerhans which constitute

approximately 2% by weight of the human pancreas (Bonner-weir et al, 2005). Several types of endocrine cells coexist in the islets. Insulin secreting beta cells represent 60-80%, glucagon secreting alpha cells are around 20%, pancreatic polypeptide containing PP cells ~15%, somatostatin delta cells ~ 10%, and ghrelin secreting cells ~1% (Wierup et al, 2002; Rhodes et al,2005). The beta cell mass is changed at different stages of life as a result of dynamic balance among replication, size modification, neogenesis, and apoptosis (Dor et al, 2004).

Although it was suggested that beta cell mass reduction is present in all type of diabetes, studies yielded discrepant results due to the limited access to human pancreas tissue, especially for type 2 diabetes (Stefan et al, 1982; Saito et al, 1978. Sakuraba et al, 2002). Recently, studies with available pancreas specimens from surgery and post-mortem samples, supported that beta cell mass is reduced in type 2 diabetes. Butler and his co-workers obtained 124 pancreatic autopsy samples from obese with/without diabetes, lean with /without diabetes and obese with impaired fasting glucose and examined their beta cell mass together with cell proliferation and apoptosis. They found obese non diabetics had a 50% increase in relative beta cell volume and an increased neogenesis from ductal tissue versus lean non diabetics; obese patients with diabetes or impaired fasting glucose were associated with a 63% and 40% deficit respectively, compared with obese non diabetics; lean patients with diabetes had a 41% less relative beta cell volume than lean controls. Another important finding in this study is that the reduction of beta cell mass is attributed to increased frequency of apoptosis other than diminished replication (Butler et al, 2003). Marchetti et al. also observed elevated apoptosis by significantly increased activity of cysteine-aspartic acid protease (caspase) 3 and caspase 8 in human diabetic beta cells, and metformin was reported to be able to

reduce cell death and normalize caspase 3 and caspase 8 activity as well (Marchetti et al, 2004).

2.3.2 Impaired Insulin Secretion Function

The reduction of beta cell mass does not solely cause the progression of type 2 diabetes because in rodents with experimental pancreatectomy the removed up to 50% of tissue still maintained normal glycemia. Therefore, alterations of insulin secretion become another interest of research. It has been extensively reported that multiple alterations of insulin release exist in type 2 diabetics which are characterized by a defective insulin response to glucose (Kahn et al, 2003; Del et al, 2002; Lin et al, 2002). Normally, glucose stimulated insulin release is biphasic process with a short early phase and much longer second phase (Henquin et al, 2000; Del et al, 2002). In type 2 diabetics, the commonly found alterations are reduced or absent first phase insulin secretion in response to intravenous glucose, blunted release in the second phase, and delayed response to an ingestion of a mixed meal (Kahn et al, 2003; Del et al, 2002). In addition, the pulsatile pattern of insulin release is disrupted instead of taking place every 8-10 minutes superimposed on low frequency oscillations (Porksen et al, 2002; Schmitz et al, 2002).

According to a well recognized hypothesis, under physiological circumstance, glucose stimulates insulin secretion through the following steps (Marchetti et al, 2008). First, glucose enters beta cell with aid from transmembrane protein glucose transport 2, and undergoes catabolism via glycolysis and the Krebs cycle, which produces reducing equivalents. The reducing equivalents then are transferred to the respiratory chain, also called the electron transport chain, to form a proton gradient across the inner mitochondrial membrane which couples the electron transport chain with oxidative

phosphorylation and results in synthesis of ATP. Secondly, augmented ATP concentrations increase the ATP/ADP ratio which leads to the closure of the ATP-regulated K^+ channels. Next the depolarization of cell membrane opens voltage-dependent Ltype Ca^{2+} channel which causes an influx of calcium ions. Finally, the elevated cytoplasmic calcium ion promotes exocytosis of insulin granules. The alterations of mitochondrial in type 2 diabetics have been indicated as one mechanism of the impaired glucose stimulated insulin secretion in addition to decreased expression of the glut2 gene and reduced activity of glucokinase (Del Guerra et al, 2005). Beta cell mitochondria in type 2 diabetes have a higher density volume and become swollen with undefined membranes (Welsh et al, 2005; Anello et al, 2005).

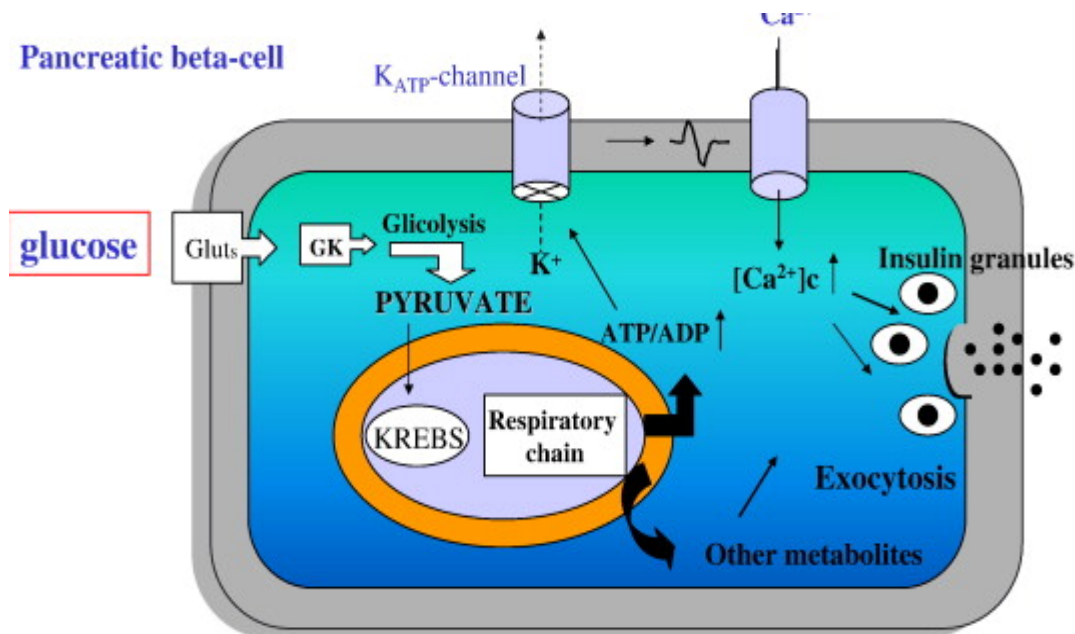


Figure 2.1 Mechanisms of glucose-induced insulin secretion (Marchetti et al, 2008)

Besides, ATP concentrations were lower as well as the ATP to ADP ratio. However, the protein expression of two mitochondrial complexes of respiratory chain, complex1 and 5, were found increased in diabetic beta cells. An increase of uncoupling protein -2 (UCP-2) protein expression was also reported in this study (Anello et al, 2005).

Taken together, it is suggested that the activated UCP-2 might be a key factor leading to lower ATP production and eventually insulin secretion impairment.

UCP-2 protein expression can be activated by superoxide (Krauss et al, 2003). Several markers of oxidative stress, nitrotyrosine and 8-hydroxy-2-deoxyguanosine, were shown higher in type 2 diabetes than control. Both of the markers were also found correlated with the severity of defective insulin secretion in response to glucose (Del Guerra et al, 2005). Other evidence indicating the involvement of oxidative stress include reduced expression of manganese-superoxide, increased expression of protein kinase c-beta2 and nicotinamide adenine dinucleotide phosphate reduced-oxidase (reactive oxygen species producing enzymes) in diabetic islet cells (Marchetti et al, 2004; Del Guerra et al, 2005). Decrease of the nitrotyrosine level and improvement of glucose stimulated insulin secretion were observed when islets of type 2 diabetes were incubated with metformin for 24 hours in vitro (Marchetti et al, 2004). Antioxidants improved impaired beta cell function and apoptosis in isolated islets exposed to elevated glucose (Kaneto et al, 1996).

Amyloidosis may play a role in the pathology of diabetes. Epidemiological studies found a high prevalence of amyloidosis in type 2 diabetes patients and a significant correlation between pancreatic amyloid formation and HbA1c (Zhang et al, 2003; Westermark et al, 1987). Pancreatic amyloid plaques formation is a feature of type 2 diabetes histopathology (Clark et al, 1987). The beta cell is almost the only cell that expresses the islet amyloid polypeptide gene. The protein is co-secreted with insulin. It is still not clear what kind of role amyloid plays in the pathogenesis of type 2 diabetes. Possibly, the high concentration of islet amyloid polypeptide which leads to the formation of amyloidosis is caused by increased production of insulin secondary to hyperglycemia (Hoppener et al, 2006; Ritzel et al, 2007).

Glucotoxicity may also be involved in diabetes. Prolonged exposure to elevated glucose was demonstrated to exert a slow but irreversible effect on beta cells which enhances apoptosis and exacerbates glucose stimulated insulin secretion (Robertson et al, 2000). Chronic hyperglycemia might decrease insulin gene transcription through its negative impact on beta-cell specific transcription factors (Kaiser et al, 2003). Also chronic oxidative stress was suggested as at least part of the mechanism underlying glucotoxicity (Tanaka et al, 1999).

Lipotoxicity is also a factor. Free fatty acids are necessary to maintain normal beta cell function; however, elevated fatty acids could inhibit islet gene expression such as GLUT-2 partially through transcript factor pancreatic-duodenum homeobox-1 (Gremlich et al, 1997). Lipotoxicity requires the presence of concomitant hyperglycemia to affect beta cell function. Accumulation of long chain fatty acyl CoAs and increased production of ceramide might mediate the deleterious effects of elevated free fatty acids (Prentki et al, 2006).

Another factor is inflammation. Increased numbers of macrophages were found in diabetic pancreatic islet (Ehse et al, 2007). Exposure to a type 2 diabetes milieu resulted in human islets to release inflammatory factors such as interleukin 6, interleukin 8, and macrophage inflammatory protein, which activated migration of monocytes and neutrophils (Ehse et al, 2007). Further research is needed to determine whether inflammation is a causal factor.

2.4 Animal Models of Type 2 Diabetes

2.4.1 General Introduction

The incidence of type 2 diabetes has reached a new record high and brings serious socio-economic burdens. Over the last years, numerous researchers have been motivated

to devote their efforts to the study of diabetes, and intense efforts have been performed which greatly advances our knowledge. Appropriate experimental models are essential to facilitate the research. The animal models of type 2 diabetes can be classified into five categories: spontaneously or genetically derived, chemicals induced, diet induced, surgical manipulation, and genetically engineered (Srinivasan et al, 2007). Due to the advantages of reduced cost, ease of maintenance and breeding, most available models are based on rodents. A list of animal models was summarized in table 2.1 and the Goto-Kakizaki Rat (GK) which was used in this dissertation would be discussed in detail.

Table 2.3 Summary of animal models of type 2 diabetes (adapted from Srinivasan et al, 2007)

Model class	Name	Obese	Origin
Genetically derived	ob/ob mouse (lep ^{ob})	Yes	Monogenic defect of leptin gene on chromosome 6 in C57BL/6J or C57BL/KS mice. Autosomal recessive mutation
	db/db mouse (lepr ^{db})	Yes	Monogenic defect of leptin receptor gene on chromosome 4 in C57BL/K _s J mice. The C57BL/K _s J - lepr ^{db/+} develops diabetes during pregnancy. Autosomal recessive mutation
	KK mouse (kuo Kondo)	Yes	Polygenic model from selective inbred for large body size in Japan

(Table 2.3 Con'd)

KK/A ^y mouse (yellow KK obese mouse)	Yes	Was developed from cross breeding of A ^y /a (dominant mutation) mice with one of the inbred KK strains
New Zealand Obese mouse (NZO)	Yes	An inbred strain of polygenic model selected for agouti coat color
Tsumara Suzuki Obese Diabetes mouse (TSOD)	Yes	Polygenic origin inbred from obese male ddY strain
Zucker Fatty Rat (lepr ^{fa})	Yes	mutation of a single autosomal recessive gene on chromosome 5 (fa)
Zucker Diabetic Rat (ZDF)	Yes (less than ZFR)	Substrain of ZFR inbred for hyperglycemia
Otsuka Long Evans Tokushima Fatty Rat (OLETF)	Yes	Polygenic rat model by selective breeding from out bred colony of Long Evans
Obese Rhesus Monkey	Yes	
Cohen Diabetic Rat	No	Special model genetically susceptible to carbohydrate-rich diet by selective inbred

(Table 2.3 con'd)

Diet induced	Goto-Kakizaki Rat(GK)	No	Polygenic model obtained from selective inbreeding of Wistar for abnormal glucose tolerance
	Akita Mouse(non obese mutant C57BL/6)	No	Autosomal dominant mutation in the insulin II gene
	Sand Rat	Yes	Develops obesity and diabetes when in captivity and fed on Chow diet (high energy compared to its low energy vegetable diet)
	C57BL/6J Mouse	Yes	Easy to induce obesity and diabetes by high fat diet. This model reflects both genetic and environmental influences
	Acomys Calirinus	Yes	When on high energy diet and placed in captivity, it gains weight and exhibits impaired glucose induced insulin secretion with marked beta cell hypertrophy.
Chemically Induced	Alloxan (ALX)	No	Induce beta cell necrosis in rabbits early in 1943. Almost replaced by STZ due to several limitations (short half-life; only stable in acid; variant success rate in inducing diabetes;

(Table 2.3 con'd)

Surgical models	Streptozotocin(STZ)	No	ketosis and mortality are high. Glucosamine derivative of nitrosourea. Low dose multiple injection or in combination with other treatments.
	Goldthiogluucose obese diabetic mouse	Yes	Targeted transported to ventromedial hypothalamus, cause necrosis and develop subsequent hyperphagia etc. after 16weeks of injection. High mortality and long duration limit its application in research
	Partial pancreatectomy	No	70-90 % dissection of pancreas. Now it is very useful in pancreatic regeneration research
	Bilateral Uterine Artery Ligation	No	Intrauterine growth retardation induced by insufficient uteroplacenta blood flow leads to hyperglycemia and impaired beta cell function in offspring. Not practical in diabetes research except for certain studies.
	Ventromedial Hypothalamus Dietary Obese Diabetic Rat	Yes	Induced by high fat high sucrose diet plus bilateral electrolyte lesion of VMH

(Table 2.3 con'd)

Genetically engineered	Particular model is generated by altering the expression of certain gene or deleting the endogenous gene in order to specifically investigate the role of the gene in development of diabetes
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2.4.2 Goto-Kakizaki Rat (GK)

The GK rat is a polygenic model of type 2 diabetes derived from Wistar rats in Japan. Goto and his coworkers performed oral glucose tolerance tests (OGTT) in 211 normal Wistar rats and selected 9 pairs of rats with the highest blood glucose levels as a foundation breeding stock in the 1970s (Goto et al, 1975). After five generations of repeated selective breeding for slightly impaired glucose tolerance, a new model of non-obese, non-insulin dependent diabetes was developed. Since the ninth generation, GK rats began to be inbred to conserve glucose intolerance and impaired glucose-induced insulin secretion (Galli et al, 1996; Östenson et al, 2001). Up to this date, besides the Japanese colony, other colonies have been established with breeding pairs from Japan worldwide, most notably in Paris and Stockholm. Optimal housing conditions with a humidity of 40~60% and temperature of 25~26 °C are essential to breed GK rats (Malaisse-Lagae et al, 1997).

The GK rat is characterized by moderate hyperglycemia, hypoinsulinemia, normolipidemia and impaired glucose tolerance which is thought to be the results primarily of reduced beta cell mass and a defective insulin response to glucose (Portha, 2005; Movassat et al, 1997; Movassat et al, 2007; Movassat et al, 1995). Extensive

studies in the Paris GK rats have shown that the beta cell deficit started at fetal age 16 days which led to a more than 50% reduction of beta cells at birth compared with normal Wistar rats (Miralles et al, 2001). Poor proliferation and enhanced apoptosis of undifferentiated ductal cells were detected in the GK fetus at embryonic age 16-20 days and the decreased pool of endocrine precursors was speculated to contribute to the defective beta cell neogenesis (Miralles et al, 2001; Calderari et al, 2007). In addition, insulin like growth factor 2 (IGF-2) production was demonstrated crippled and might account for the retarded beta cell growth (Serradas et al, 2002). Interestingly, maternal food restriction (65% restriction) during the last week of gestation improved beta-cell mass and pancreatic IGF-2 levels in GK fetuses, indicating an epigenetic effect (Fernández-Millán et al, 2009). After birth, GK rats present continuously less beta cell mass. The total beta-cell mass was only 35% of that in age matched control pancreases on day 4, 30% on day 7 and 37% on day 14 (Movassat et al, 1997). The pancreatic insulin content was also reduced to as low as 31–40%. Despite this, during this period, GK neonates exhibited normal basal plasma glucose and glucagon levels until they were 4 weeks old. When the GK rats reach 8 weeks of age, they exhibit marked hyperglycaemia and slightly low fasting plasma insulin with total beta cell mass reduced by more than 60% (Movassat et al, 1997). The loss of beta cell mass was ameliorated by the glycemia lowering drugs glucosidase inhibitor or GLP-1/exendin-4 in adult and postnatal GK rats (Koyama et al, 2000; Turrel et al, 2002).

The islet morphology of GK rats is characterized by the presence of ‘starfish shaped’ islets. In the pancreas of normal Wistar rat, beta cells form a core localized in the center of the islet whereas non-beta cells envelop a peripheral, continuous mantle. The boundary is well defined (figure 2.2). In GK rats, the ‘starfish shaped’ islet is

featured by irregular capsules with disrupted architecture caused by transverse strands of connective tissue (figure 2.3). The changes in islet morphology are rare in neonatal and young GK rats, but increase as the animal ages (Suzuki et al, 1992). Several anomalies were observed existing in the ‘starfish shaped’ islets including inflammatory marker such as major histocompatibility complex (MHC) II and macrophage-associated antigens (CD68), overexpression of IGF-2, and these might partially account for the changes that occur (Homo-Delarche et al, 2006; Hoog et al, 1996; Hoog et al, 1997).

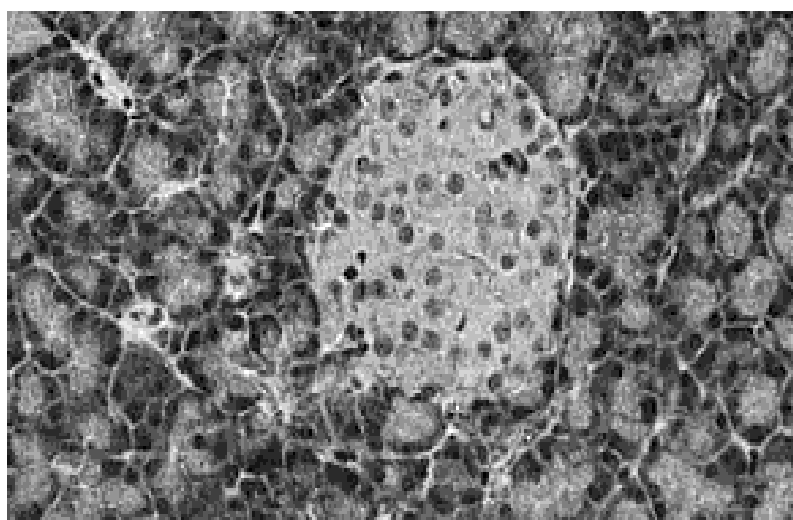


Figure 2.2 Normal shaped islet (Shafrir, 2007)

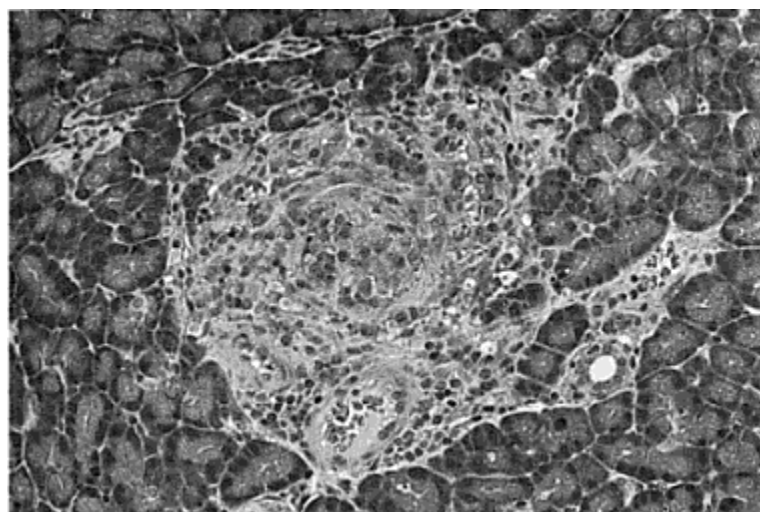


Figure 2.3 Starfish shaped islet (Shafrir, 2007)

The GK rat was generated by selective inbreeding which indicates its hereditary nature. From cross-breeding studies with GK and Wistar, it suggested that genes from both parents are necessary for the manifesting of the GK rat to be fully expressed in the offspring (Serradas et al, 1998). As a polygenetic model of diabetes multiple genes are involved in the developing of diabetic features in the GK rat. To present, several loci were found susceptible to glucose intolerance and impaired insulin secretion through linkage analysis (Galli et al, 1996; Gauguier et al, 1996). Niddm1/Nidd/gk, which resides on chromosome 1, causes defective insulin secretion and is regarded as a main contributor to postprandial hyperglycemia. Another less important locus Niddm2/Nidd/gk2 is located on chromosome 2 and influences both fasting and postprandial glycemia. Weight1.bw/gk1 is on chromosome 7 and is linked to body weight. Although currently there is no diabetic gene that has been identified in the aforementioned chromosomal loci, genes coding for mitochondrial glycerol-3-phosphate dehydrogenase, insulin-degrading enzyme, and uncoupling protein 2 were suggested as candidates (Fakhrai-rad et al, 2000; Koike et al, 1996; Kaisaki et al, 1998). However, so far no locus associated with beta cell mass has been found, and the beta cell mass was reported intact in Niddm1 subcongenics (Granhall et al, 2006), which raises a question about whether the genotype alteration is directly linked to low beta cell mass.

There is a growing body of evidence that indicates that impaired beta cell function is another major pathologic character present in pancreatic islet of GK rats. Compared with islets from normal Wistar, those from GK rats showed inappropriately reduced insulin secretion in response to glucose (Mosén et al, 2005; Galli et al, 1996; Mosén et al, 2008). It is generally accepted that glucose induces insulin secretion through following steps. First, glucose enters the beta cell with aid from transmembrane protein glucose

transport 2, then produces ATP after a series of reactions (glycolysis and Krebs cycle). Secondly, the augmented ATP concentration increases the ATP/ADP ratio which leads to the closure of the ATP-regulated K^+ channels. Next the depolarization of the cell membrane opens voltage-dependent L-type Ca^{2+} channel which causes an influx of calcium ions. Finally, the elevated cytoplasmic calcium ion promotes exocytosis of insulin granules. Any defects in this pathway will result in the diminished insulin secretion response to glucose. Glucose transport 2 was found underexpressed in GK rats, but this is unlikely to be the only causal factor (Ohneda et al, 1993). Several defects in the main enzymes that catalyzed oxidative glycolysis have been reported which consisted of reduced activity of FAD linked glycerol phosphate dehydrogenase (Fabregat et al, 1996), pyruvate dehydrogenase (Zhou et al, 1995), and pyruvate carboxylase (MacDonald et al, 1996). However, the decreased enzyme activity was restored when glucose was normalized by insulin (MacDonald et al, 1996). In addition, over expression of FAD linked glycerol phosphate dehydrogenase in GK rats didn't correct altered insulin secretion (Ueda et al, 1998). Other possible mechanisms are suggested such as dysfunction of lysosomal glycogenolytic enzymes (Salehi et al, 1999), anomaly of glucose-heme oxygenase-carbon monoxide signaling pathway (Mosén et al, 2005), reduced NADH/NAD ratio by enhanced activity of adenylyl cyclase III (Abdel-Halim et al, 1993) which further studies are needed to verify.

Arginine, a nonglucose insulin secretagogue, was shown to induce an increased pancreatic insulin response in GK rat (Hughes et al, 1994; Abdel-Halim et al, 1993). However, Portha and his co-workers reported that in the absence of glucose arginine failed to induce a normal insulin response in GK rats as it did in the control rats (Portha

et al, 1991). Therefore, arginine stimulated insulin secretion might also be disturbed in GK rats.

It has been proposed that the reduced beta-cell mass and impaired function in the GK model are ascribed to an interaction of multiple pathogenic factors: (i) defects in several genes account for weakened insulin secretion; (ii) decreased beta-cell neogenesis and/or proliferation due to an epigenetic influence of gestational metabolic programming in the pancreas; and (iii) secondary loss of beta-cell differentiation due to chronic exposure to hyperglycemia (Portha et al, 2009; Portha, 2005).

Insulin sensitivity in GK rat has been studied using the hyperinsulinemic-euglycemic clamp in vivo and in tissues such as liver, muscle and adipose tissue in vitro. Suzuki et al. utilized the clamp in combination with a tracer to show that the dysregulation of hepatic fructose-2,6-bisphosphate and subsequent elevation of hepatic glucose production was the main reason for mild insulin resistance in GK rats (Suzuki et al, 1992). Other defects featured by a decrease of receptor number with normal tyrosine kinase activity and attenuated inhibition of insulin on glucagon-induced hepatic glucose production were also observed (Doi et al, 2001). In skeletal muscle, the blunted conversion of glucose to glycogen due to chronic activation of protein kinase C was regarded as one of the contributors to insulin resistance (Avignon et al, 1996). In addition, several defects in the postreceptor insulin signaling pathway were demonstrated in adipose tissue and skeletal muscle that included the impaired insulin – stimulated tyrosine phosphorylation of insulin receptor substrate-1 (Begum et al, 1998), and attenuated PI3K activated Akt kinase (Krook et al, 1997). Interestingly, the Akt kinase activity was able to be restored after nearly normalization of glycemia in GK rats, which suggested that the impaired insulin sensitivity in extrahepatic tissues is probably

secondary to hyperglycemia (Krook et al, 1997). These observations as well as the pathological progress in insulin-mediated muscle glucose transport with aging indicate the supporting role of insulin resistance and the leading effect of a beta-cell secretory defect in the development of diabetes in the GK rats.

2.5 Methods of Measuring Insulin Sensitivity

Systemic (whole body) insulin resistance is determined by insulin sensitivity in the insulin target tissues, which include muscle, heart, liver, and fat. Muscle insulin resistance is a major portion of systemic insulin resistance. Several factors are known to induce insulin resistance. These include obesity, lipodystrophy, genetic background, inflammation, free fatty acids/intermediates, ectopic fat in muscle, endoplasmic reticulum stress, oxidative stress, hyperinsulinemia, mitochondrial dysfunction and aging.

The insulin resistance can be measured with a combination of markers that represent characteristics of insulin resistance. These include hyperinsulinemia, decreased glucose infusion rate, hyperglycemia, impaired glucose tolerance and so on.

2.5.1 Direct Measures

2.5.1.1 Euglycemic Hyperinsulinemic Clamp

The euglycemic hyperinsulinemic clamp is regarded as the "gold standard" for measuring whole body insulin sensitivity (Muniyappa et al, 2008). It directly measures glucose utilization promoted by insulin in the steady state. In this method, insulin is infused at a constant rate after an overnight fast to raise blood insulin concentration to certain level that is higher than that in the fasting phase, which is the origin of 'hyperinsulinemia'. This hyperinsulinemia results in suppression of hepatic glucose production and increase of glucose disposal in skeletal muscle and adipose tissue. In the meanwhile, 20% dextrose is administered intravenously to maintain euglycemia which is

monitored by frequently using glucose analyzed every 5 to 10 minutes. Considering that there is no net glucose change in the steady state given that the hyperinsulinemia can suppress hepatic glucose production completely, the glucose infusion rate (GIR) that is needed to maintain euglycemia status would be equal to the glucose disposal rate. However, the clamp method is based on assumptions: 1. hyperinsulinemia suppresses hepatic glucose production entirely; 2. a steady state condition is achieved at the end of the procedure; 3. insulin infusion rate is properly chosen for the target population in terms of insulin sensitivity. GIR is usually needed to be normalized for fat-free mass. Sometimes GIR is adjusted for the blood glucose concentration in the steady state condition, and the difference in insulin concentration between the fasting stage and the steady state condition where is formulated as $GIR / (G \times \Delta I)$. This is the insulin index derived from clamp.

The clamp requires blood sample collection, and the procedure per se is very complicated and time consuming, which limits its application in large-scale human and animal studies.

2.5.1.2 Insulin-suppression Test (IST)

The insulin-suppression test is another method besides the clamp that directly measures metabolic insulin sensitivity/resistance. It measures the ability of exogenous insulin to drive glucose utilization in peripheral tissues under steady-state circumstance provided with complete inhibition of endogenous insulin secretion (Harano et al, 1978). In this procedure, patients fasted overnight receive i.v. somatostatin at a constant rate (250 µg/h) or the somatostatin analogue octreotide (25 µg bolus, followed by 0.5 µg/min) to achieve a full suppression of endogenous secretion of insulin and glucagon. Simultaneously, sustained infusions of exogenous insulin (25 mU/m²/min) and glucose

(240 mg/m²/min) into the same antecubital vein over 3 h are performed to obtain steady states of plasma insulin and plasma glucose. Blood samples from the other forearm are collected every 30 minutes for the first 150 minutes and then every 10 minutes for a half an hour after initiation of the infusions. The second period is usually considered as the steady –state phase. The steady-state plasma glucose (SSPG) concentration and steady-state plasma insulin (SSPI) concentration are determined at this stage. Considering that the universal insulin infusion rate for all subjects would result in generally similar SSPIs, insulin sensitivity is inversely related to the SSPG values. The formula calculated in IST is $ISI (dl \cdot kg^{-1} \cdot min^{-1}) = [glucose \text{ infusion rate } (mg \cdot kg^{-1} \cdot min^{-1}) / SSPG (mg/dl)] \times 103$. Similar as the clamp, IST is based on several assumptions: 1. somatostatin infusion is sufficient to suppress endogenous secretion of insulin and glucagon; and 2. hepatic glucose production is completely inhibited. Although IST has its advantages over clamp such as it is easier to achieve a steady-state condition, less labor intensive, it is still not practical in the clinical care setting and large studies. And some errors may be introduced in cases like type 2 diabetics and extremely insulin sensitivity individuals.

In addition to the aforementioned direct measures of insulin sensitivity, two indirect measures are presented below.

2.5.2 Indirect Measures

2.5.2.1 Minimal Model

Minimal model is a mathematical model with 2 coupled differential equations and 4 parameters. The first equation represents plasma glucose dynamics and the second equation describes insulin dynamics in two separated compartments.

It allows calculating insulin sensitivity in a dynamic state on the basis of concentrations of glucose and insulin collected from a “frequently sampled intravenous

glucose tolerance test” (FSIVGTT). During this 3-hour procedure, a bolus of glucose (0.3g/kg body weight) is administered intravenously over 2 minutes in an overnight fasted patient at time 0. Blood samples are collected for plasma insulin and glucose which are used to generate the index of insulin sensitivity (S_I) and glucose effectiveness (SG) and obtain information about β -cell function (Bergman et al, 2002). S_I is defined as fractional glucose disappearance per plasma insulin unit, and SG is the ability of glucose to inhibit hepatic glucose production and mediate glucose disposal independent of increased insulin. In humans, the peak insulin induced by FSIVGTT overlaps the glucose effectiveness period, as well as the inadequate endogenous insulin secretion in diabetics, a modified FSIVGTT was developed with infusion of exogenous insulin or tolbutamide over 5 minutes at 20 minutes after the glucose bolus (Quon et al, 1994; Saad et al, 1997).

This minimal model analysis has been demonstrated to be comparable to the clamp in healthy subjects (Beard et al, 2007), and has been used in relative large scale study (Howard et al, 1996). However, it lumps together insulin’s ability of suppressing hepatic glucose production and accelerating glucose disposal, so it is less accurate in insulin resistant subjects.

2.5.2.2 Oral Glucose Tolerance Test/Meal Tolerance Test

Being abbreviated as OGTT/MTT, it is a dynamic test to measure glucose intolerance and obtain indirect information of insulin sensitivity when combined with other assay. The procedure is much simpler and is widely applied in the clinical setting to diagnose type 2 diabetes (ADA, 2007) After an overnight fast, blood is drawn for glucose and insulin at 0, 30, 60, and 120 minutes preceded by an oral 75 gram glucose load or standard meal. It is important to note that, glucose tolerance is a comprehensive outcome

involving insulin secretion, other hormones, and insulin sensitivity. It is not equivalent to insulin sensitivity.

Simple surrogate measures of insulin sensitivity have been proposed and assessed against the clamp.

2.5.3 Surrogate Indexes from Fasting Steady State

2.5.3.1 HOMA Model

The homeostatic model assessment (HOMA) uses a set of empirically derived nonlinear equations to estimate insulin resistance and beta-cell function from fasting glucose and insulin data. The approximation equation yielded from this model to determine index of insulin sensitivity is HOMA-IR: $\text{Glucose (mmol/l)} \times \text{Insulin } (\mu\text{U/mL}) / 22.5$. The result calculated from this equation has a good linear correlation with results from the euglycemic clamp method (Radziuk, 2000)

2.5.3.2 Quantitative Insulin Sensitivity Check Index (QUICKI)

QUICKI is another mathematical equation used to estimate insulin sensitivity based on a log transform of the product of the fasting blood glucose and plasma insulin concentrations. It was developed upon the discovery that the data from the first 20 minutes of an FSIVGTT was adequate to generate a reliable index for insulin sensitivity as that determined by a reference glucose clamp (Katz et al, 2000). Additional log transformation was taken to maintain linear correlation with the clamp in diabetics. QUICKI showed better linear correlation with the clamp than the HOMA and minimal model methods (Cobelli et al, 1998; Katz et al, 2000). QUICKI, as a simple, accurate index of insulin sensitivity as well as one of the most thoroughly evaluated is appropriate for large scale studies.

$$\text{QUICKI} = 1 / [\log(\text{fasting insulin, } \mu\text{U/ml}) + \log(\text{fasting glucose, mg/dl})]$$

2.5.4 Surrogate Indexes from Dynamic Test

Based on glucose and insulin data from OGTT, IVGTT, MTT, or OGTT/MTT combined minimal model, a number of indexes have been developed, such as the Insulin sensitivity index-Matsuda, Avignon index, oral glucose insulin sensitivity index, and Gutt index. Many of these indexes correlate well with the glucose clamp estimates of SI (Gutt et al , 2008; Mari et al, 2001; Matsuda et al, 1999). The advantage of the dynamic test based surrogates is to be able to get both data about insulin secretion and insulin action at same time. However, the decision of choosing fasting surrogates or dynamic surrogates depends on research interest.

$$\text{ISI(Matsuda)} = 10,000/\sqrt{[(G_{\text{fasting}} \times I_{\text{fasting}}) \times (G_{\text{OGTTmean}} \times I_{\text{OGTTmean}})]}$$

Gutt index= $\text{MCR}/\log \text{MSI} = \text{m}/\text{MPG}/\log \text{MSI}$. The glucose uptake rate in peripheral tissues $\text{m} = [75,000 \text{ mg} + (\text{Glucose}_0 - \text{Glucose}_{120}) \times 0.19 \times \text{BW}]/120 \text{ min}$, The metabolic clearance rate (MCR) = m/MPG (mean of the glucose concentration at 0 and 120-min of the OGTT). MSI stands for mean serum insulin (mU/l) is the mean plasma insulin concentrations at 0- and 120-min of the OGTT.

2.5.5 Measures in Animal

As in human, the euglycemic-hyperinsulinemic clamp is also the ‘gold standard’ for measuring whole body insulin sensitivity in animals. However, especially in rodents, due to the lack of true physiologic fasting state and a small blood volume, the clamp is not easy to conduct. Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) are two frequently used methods of measuring glucose tolerance and insulin sensitivity in rodents (Gao et al, 2009). Although they can’t estimate insulin sensitivity as precisely as the clamp does, they are still the method of choice when insulin sensitivity is of secondary interest or the clamp is infeasible.

In animals, HOMR-IR and QUICKI also have been validated by examining correlations with the reference glucose clamp method (Cacho et al, 2008; Lee et al, 2008). The equation is HOMA-IR: $\text{Glucose (mg/dl)} \times \text{Insulin } (\mu\text{U/mL}) / 2430$. Overall, these two surrogates provide a reliable and simple estimate of formal measures of insulin sensitivity with variability and discriminant power comparable to the clamp when applied to rats and mice (Cacho et al, 2008).

2.6 Adiponectin

Adiponectin is a 244- amino acid protein encoded by gene located on chromosome 3q26 (Ruan et al, 2003). It is produced mainly by white adipose tissue, trace by brown adipose tissue, liver, muscle etc. (Vienngchareun et al, 2002). Adiponectin is the most abundant adipose tissue derived hormone and the average plasma concentration in humans ranges from 5~30 ug/ml, about 1000 times more than leptin (Berg et al, 2002). In the circulation adiponectin exists in multimers, high-molecular-weight isoform, medium-molecular-weight hexamers, and low-molecular-weight trimers, among which the high-molecular-weight is proposed to be more biologically active than other forms (Heidemann et al, 2008; Lara-Castro et al, 2006). Adiponectin has a profound insulin sensitizing effect as well as anti-inflammatory and antiatherogenic effects. It also promotes food intake as the result of action in the hypothalamus (Kubota et al, 2007). The plasma adiponectin level is found negatively correlated with visceral adiposity, insulin resistance, type 2 diabetes, and cardiovascular disease (Aso et al, 2006; Li et al, 2009). However, in the growing stage, adiponectin is initially increased with the accumulation of adiposity and then begins to decrease as the mice reach adulthood (Ziemke et al, 2010). A diet rich in whole grain, consumption of nuts, coffee and moderate alcohol (Mantzoros et al, 2006), peroxisome proliferator-activated receptor

agonist (Combs et al, 2002; Tonelli et al, 2004) and physical training (Bluher et al, 2006; Bluher et al, 2007) are shown to be associated with an increase of plasma adiponectin concentrations.

Two 7-transmembrane proteins with external C terminal and internal N terminal regions have been identified as receptors for adiponectin (Yamauchi et al, 2003). Although both receptors are globally present, Adipo R1 is highly expressed in muscle while Adipo R2 is dominant in the liver. AdipoR1 knockout mice exhibit increased adiposity, decreased glucose tolerance, physical activity, and energy expenditure. On the contrary, AdipoR2 deficiency resulted in increased physical activity and energy expenditure, lowered plasma cholesterol levels, and an enlarged brain size. These mice are resistant to HFD-induced obesity and glucose intolerance (Bjursell et al, 2007). The adiponectin concentration in the blood stream is in proportion to the expression of Adipo R2 in subcutaneous fat whereas it is negatively related to the expression of Adipo R1/R2 in muscle; however, in insulin resistance the expression of the receptors in both location was increased (Bluher et al, 2006; Bluher et al, 2007).

Adiponectin mainly signals through AMP-activated protein kinase to exert its beneficial effects on the above mentioned diseases after binding to the receptors (Yamauchi et al, 2002). Several other signaling pathways are also proposed as mediators. Pathways involved include mTOR, nuclear transcription factor-kB, STAT3, and JNK (Averous et al 2006; Miyazaki et al, 2005; Tomas et al, 2002).

Accumulating evidence supports linkage between altered circulating adiponectin and insulin resistance /type 2 diabetes. Lower plasma total adiponectin was not only shown associated with increased insulin resistance (Stefan et al, 2002), but also was found in type 2 diabetics compared with BMI- matched controls (Hotta, et al, 2000). In a

meta-analysis including thirteen prospective studies with a total of 14 598 subjects and 2623 type 2 diabetics, it was observed that adiponectin levels were inversely associated with the risk of developing type 2 diabetes. The relative risk of type 2 diabetes for each one unit increment of adiponectin was 0.72 indicating a protective role of adiponectin in the occurrence of type 2 diabetes (Li et al, 2009). Animal studies also were in accordance with observations in humans. Adiponectin knockout mice showed notable insulin resistance on a high fat, high sucrose diet; a supplement of adiponectin improved this insulin resistance (Maeda et al, 2002). A similar insulin resistance reversal effect was reported when physiologic doses of adiponectin were replenished to a lipotrophic mouse model (Yamauchi et al, 2001).

In most epidemiological studies it is the total immunoreactive adiponectin that was assessed rather than the multimeric forms of adiponectin. One study reported that although the total adiponectin was significantly related to glucose intolerance, the ratio of high molecular weight to total was a tighter indicator in Indo-Asian males (Fisher et al, 2005). However, contradictory results have shown that total adiponectin was not correlated with insulin sensitivity whereas the ratio of high molecular weight to total was positively correlated (Pajvani et al, 2004). Lara-Castro et al found total adiponectin, high molecular weight, low molecular weight, and high to total ratio were all significantly associated with the insulin stimulated glucose disposal rate. Moreover, all these indicators were positively correlated with reduced central fat distribution and augmented fat oxidation rate. Regarding the lipoprotein profile, high molecular weight and total were related to favorable changes. After further analysis, the authors concluded the quantity of high molecular weight was the primary factor for all these relationships (Lara-Castro et al, 2006).

Maternal circulating adiponectin was also found lower in pregnant women with gestational diabetes (Cseh et al, 2004; Ranheim et al, 2004; Kinalski et al, 2005; Ategbo et al, 2006;). Low plasma adiponectin is also proposed as a risk factor for gestational diabetes. Women with adiponectin concentrations less than 6.4 ug/ml at the 13th week of gestation had a 4.6-fold increased risk of developing gestational diabetes in later pregnancy (Williams et al, 2004). A recently published study explored the distribution of adiponectin multimers in gestational diabetes patients (Mazaki-Tovi et al, 2009). The results revealed that maternal serum of total, high, medium, low, and high/total were decreased in pregnant women with GDM compared to weight matched normal pregnant controls. Furthermore, all multimers were not different between obese/overweight GDM patients and normal weight GDM patients, albeit the former usually have lower concentrations than the latter without the presence of GDM. In addition, interventions such as insulin and glyburide displayed no impact on the concentrations and relative distribution of adiponectin multimers.

Patients with coronary heart disease had lower plasma adiponectin levels than age and BMI matched controls (Ouchi et al, 1999). In a case control study conducted in male patients, it was shown that an adiponectin concentration less than 4ug/ml was related to a 2 fold increase of incidences of coronary heart disease and it was independent of other risk factors (Kumada et al, 2003). Additionally, high adiponectin levels were indicated as having an inverse association with occurrences of acute myocardial infarction in men (Pischon et al, 2004). In cardiac hypertrophy caused by pressure overload in three types of mice, adiponectin-deficient, wild-type and diabetic db/db mice, a supplement of adiponectin conveyed by adenovirus attenuated the severity of the condition, and the inactivation of AMPK blunted these effects (Shibata et al, 2004). Furthermore,

adiponectin prevented plaque rupture by increasing both RNA and protein expressions of tissue inhibitor of metalloproteinase in macrophages through the promotion of interleukin-10 synthesis. Tissue inhibitor metalloproteinase can stabilize plaque by suppressing matrix metalloproteinase (Kumada et al, 2004). Adiponectin also inhibited vascular cell adhesion molecule-1 and intracellular adhesion molecule-1, thereby prevented monocytes from binding to vascular endothelial cells (Ouchi et al, 2000). Besides, adiponectin suppressed smooth muscle cell migration and proliferation in vascular remodeling by inhibiting mitogen activated protein kinase (Arita et al, 2002). Adiponectin shows protective features on cardiovascular disease as positively correlated with favorable lipid profile (high HDL and low apoB100, triglycerides) (Schulze et al, 2005) and facilitating endothelial nitric oxide synthesis (Chen et al, 2003).

2.7 Physiological Role of UCP-1

2.7.1 Coupling and Uncoupling

The mitochondrion is a membrane-enclosed organelle in most eukaryotic cells (Henze et al, 2003). It is composed of compartments which include an outer membrane, inner membrane, intermembrane space, cristae and matrix. The small molecules such as glucose and ions are able to permeate the outer membrane freely. However, the inner membrane is nearly impermeable to all molecules since it does not contain porins (Herrmann et al, 2000). The notable membrane potential across inner membrane is established by the electron transport chain.

Electron transport chain is located in the inner membrane. It contains Complex I, II, III and IV. In Complex I (NADH dehydrogenase or NADH: ubiquinone oxidoreductase), two electrons are delivered from NADH to ubiquinone (Q) which is a lipid-soluble carrier and can diffuse freely in the membrane. Simultaneously, Complex I translocates

four protons from matrix to intermembrane space across the inner membrane. Complex II (Succinate dehydrogenase or succinate-coenzyme Q reductase) is involved in both citric acid cycle and the electron transport chain (Oyedotun et al, 2004). It catalyzes succinate oxidation to fumarate. The electrons derived from succinate oxidation were transferred to an ubiquinone, which reduces the ubiquinone to an ubiquinol (QH₂). Complex III (coenzyme Q : cytochrome c — oxidoreductase or cytochrome bc₁ complex) delivers two electrons from QH₂ to cytochrome C. Concomitantly, four protons are pumped from the mitochondrial matrix into the intermembrane space (Crofts et al, 2004;Kramer et al,2004). Complex IV (cytochrome c oxidase) transfers four electrons from four molecules of cytochrome c to oxygen and produces two molecules of H₂O. Simultaneously, four protons are pumped across inner membrane to intermembrane space.

With continuously pumping protons from the matrix to the intermembrane space by complex I, III and IV, a proton gradient or membrane potential across the inner membrane is formed. This gradient is used by ATP synthase, which also locates in the inner membrane, to produce ATP through oxidative phosphorylation. Accumulated protons in the intermembrane space return to the matrix through ATP synthase which acts similar to an ion channel. Free energy released by the proton reflux fuels the ATP synthesis (Boyer et al, 1997; Yoshida et al, 2001). During the process, the electron transport chain is coupling with oxidative phosphorylation via its formed proton gradient.

In addition to ATP synthase, accumulated protons in the intermembrane space are able to return to the matrix through some other ion channels such as uncoupling protein1 (UCP-1) (Kozak et al, 1998; Nicholls et al, 1978). The uncoupling process produces lots of heat instead of ATP like a short circuit.

2.7.2 Thermogenesis of UCP-1

UCP-1 (Thermogenin) is highly present in brown adipose tissue which is only found in mammals. The main activity of UCP-1 is to generate heat via non-shivering thermogenesis, which plays an important role in heat generation of hibernating and newborn mammals (Rial et al, 2009). The mitochondrial proton circuit caused by UCP-1 leads to fast substrate oxidation without ATP production. Fatty acids are the major messengers to activate UCP-1, which is inhibited by purine nucleotides.

Brown adipose tissue (BAT) is scattered in the body such as interscapular, surrounding the kidneys and aorta (Mattson et al, 2010). The heat generated by UCP-1 is transferred to the body easily since BAT is much vascularized (Smith et al, 1964). Cold exposure is able to increase vascularization of BAT via stimulating angiogenesis (Asano et al, 1999). That will increase the heat diffused through the body.

2.7.3 UCP-1 and Diabetes

Diabetic rats induced by alloxan injection had significantly decreased UCP-1 expression (Vasilijevic et al, 2010). A leucine-deficient diet reduced abdominal fat mass in mice. That may be mediated by induced expression of UCP-1 in BAT (Cheng et al, 2010). IKKepsilon knockout mice are protected from high-fat diet-induced obesity and whole-body insulin resistance. These mice show increased energy expenditure and thermogenesis via enhanced expression of the UCP-1 (Chiang et al, 2009).

Recently, substantial depots of BAT were identified in a region extending from the anterior neck to the thorax (Cypess et al, 2009). UCP-1 was detected in the BAT. The amount of BAT is inversely correlated with body-mass index, especially in older people, and age, indicating a potential role of brown adipose tissue in the adult human metabolism.

2.8 Gut Microbiota and Metabolism

2.8.1 Normal Human Gut Microbiota

The human gastro-intestinal tract contains numerous microorganisms, known as ‘microbiota’, of which bacteria are the most dominant and largely diverse. The gastro-intestinal tract flora is essential in maintaining normal gut function and host’s health, such as forming a physical barrier which is critical in the development of mucosal and systemic immune systems and salvaging energy from dietary compound escaping digestion in the upper gastro-intestinal tract. Studies using germ free mice have shown a lack of gut microbiota made the animal more susceptible to infection, to having reduced digestive enzyme activity, and have less serum immunoglobulin levels (Shanahan et al, 2002). However, the digestive system microbiota also consists of potentially pathogenic bacteria.

The composition of the gut microbiota is so complicated and individualized that our understanding was largely limited by technical issues until the 16S ribosomal RNA gene based approaches facilitated the process. There is a growing body of evidence that the indigenous gut microbiota is established during the first year of life (Xu et al, 2003; Gronlund et al, 1999; Favier et al, 2002; Midtvedt et al, 1992). In the uterus the gastro-intestinal system of a fetus is germ free. At birth as the infant is exposed to the bacteria from the mother’s vagina, feces, skin and the environment, the intestinal colonization commences and progresses dramatically from sterility to broad mixtures of microbes (Edwards et al, 2002; Xu et al, 2003). The colonization is influenced by several factors, such as the mode of delivery, diet and environmental hygiene level (Gronlund et al, 1999). Cesarean infants have a delayed colonization with *Bifidobacterium spp* and *Lactobacilli spp* up to 30 days whereas vaginally delivered infants normally start colonization with

Bifidobacterium spp 3-4 days after birth (Kurokawa et al, 2007). Moreover, cesarean infants have a reduced number of bacteria compared with naturally delivered infants (Morelli, 2008). *Bifidobacterium spp* and *Enterobacteriaceae* are two early colonizers. Breast-fed infant digestive tracts are dominated with *Bifidobacterium spp*, whereas *Enterobacteriaceae* is prominent in formula-fed infant digestive tracts along with *Bacteroides spp* and the *Clostridia spp* family (Tannock et al., 1990; Mountzouris et al, 2002). Besides, the addition of solid food to the breast-fed infant caused a significant change in composition of the gut flora; however, it didn't have the same impact in the formula-fed infant (Stark et al, 1982). Gestational age is another factor influencing colonization. Studies show premature infants underwent delayed colonization with more virulent species (Kosloske et al, 1994; Orrhage et al, 1999).

During the weaning stage and thereafter, intestinal microflora changes rapidly and it takes up to 24 more months for the gut microbiota to complete the transformation to adult-type with unique and diverse groups of flora (Zoetendal et al, 1998). After the adult-type microbiota is formed, it remains quite constant during a considerable period of lifespan (up to 7th decade), with some fluctuation around the core colonies (Ley et al, 2006). Upon aging, the composition begins to change with a dramatic decrease of protective bacteria i.e. Bifidobacterial species and increase of enterobacteria which are considered detrimental bacteria (Mitsuoka et al, 1982; Hopkins et al, 2001). The ratio of Firmicutes/Bacteroidetes was also reported increased with age as well as the *Bacteroides* species richness; despite the age-related reduction in the *Bacteroides* number (Mariat et al, 2009).

In the adult, the microbial community residing in the human body outnumbers total host cells by ten-fold, and consists of at least 10^{14} bacteria which belong to up to

1000 different species(Cani et al, 2007). The microbiome is about 100 times more than the human genome (Kurokawa et al, 2007). Although the microbiota in the gastro-intestinal tract has large variability between individuals (Eckburg et al, 2005), the predominant bacteria divisions are relatively constant, and the majority of the bacterial population (>90%) is anaerobes. The dominant bacterial phyla are Firmicutes, Bacteroidetes and Actinobacteria (Backhed et al, 2005). Firmicutes contains over 200 genera. It is the largest bacterial phylum in the adult human gastro-intestinal tract. Species belonging to this phylum include Lactobacillus, Mycoplasma, Bacillus, Eubacterium and Clostridium (Zoetendal et al, 2006; Backhed et al, 2005). The Bacteroidetes includes about 20 genera among which Bacteroides is potent for digesting otherwise indigestible dietary polysaccharides in the distal intestinal habitat of adult humans (Xu et al, 2003). Actinobacteria and Firmicutes are gram-positive whereas Bacteroidetes is gram-negative; fluorescent in situ hybridization (FISH) rather than RNA gene sequencing is currently the only technique to detect Actinobacteria (Zoetendal et al, 2006).

As mentioned above, the gut microflora differs remarkably from one person to another. Among multiple host and external factors which influence the composition of the core microbiota, genetic makeup is the most important determinant. Studies demonstrated that identical twins have greater similarity of gut microbiota than those of unrelated individuals, even though the twins live separately (Turmbaugh et al, 2009). To the opposite, biologically unrelated people who spent life together for a long time didn't show a significant resemblance in the composition of gut bacterial communities (Zoetendal et al, 2001).

The distribution of the bacterial population is uneven in different parts of the gastro-intestinal tract. The large intestine has the largest number of bacteria with an average of 10^{11} - 10^{12} , and most of them are anaerobes from three prominent divisions in human gut. The mouth contains around 10^{10} microflora with more than 500 bacterial species and is the habitat of the second largest population of bacteria (Tlaskalova-Hogenova et al, 2004). Most of the bacterial species are hindered by the acid digestive fluid and pancreatic secretions in the stomach and proximal intestine. There are about 10^1 - 10^3 bacteria present in the stomach among which the most common microbe is Gram negative *Helicobacter pylori* (Tlaskalova-Hogenova et al, 2004).

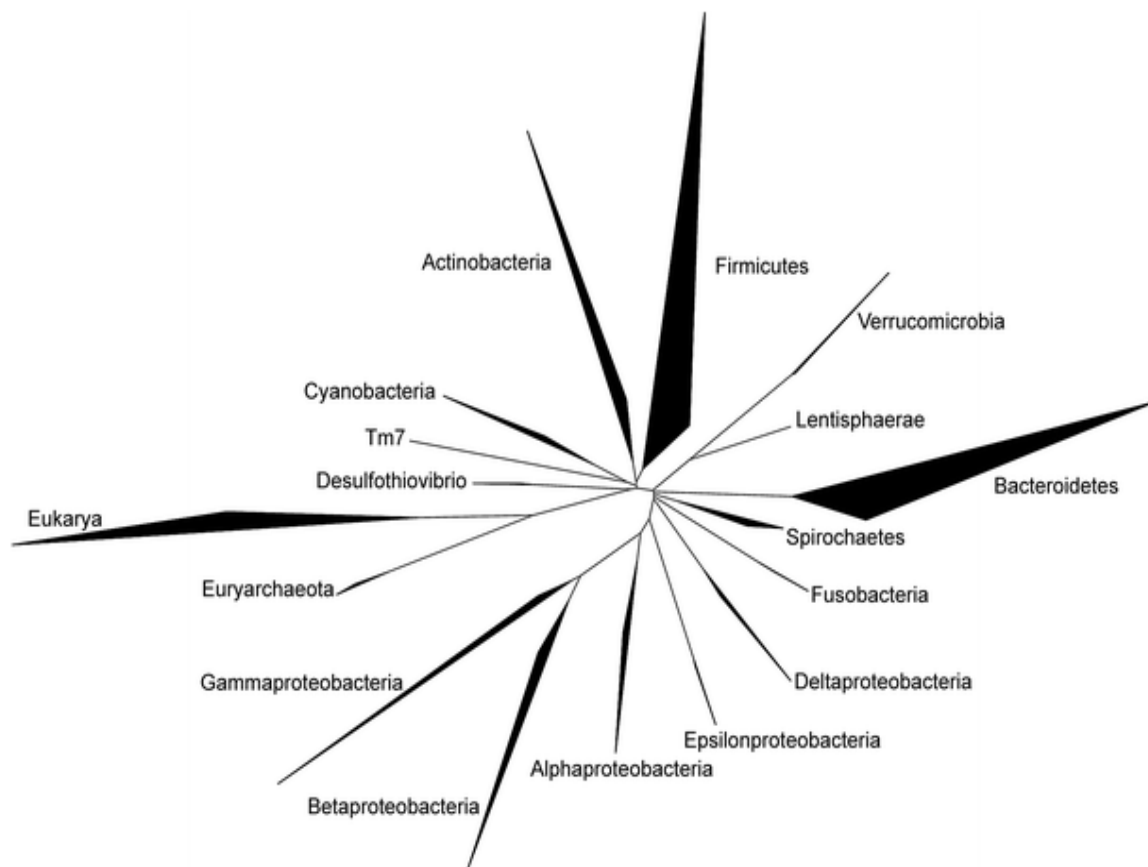


Figure 2.4 The phylogenetic tree of the most frequently detected phylotypes in human faeces using 16S rRNA gene sequencing (Vrieze et al, 2010)

It is an opportunistic pathogen and regarded as a contributor to gastric ulcers and gastric cancers. Although the small intestine harbors 10^4 - 10^7 bacteria, which the majority is Firmicutes such as *Lactobacilli*, *Bacilli* and Gram positive Cocci, it is usually considered 'relatively sterile' due to a number of nonimmune and immune factors such as pancreatic juice, constant movement, and secretory immunoglobulin A (Kleinman et al, 2008). Figure 2.5 visualized the distribution of bacteria in various parts of the gut.

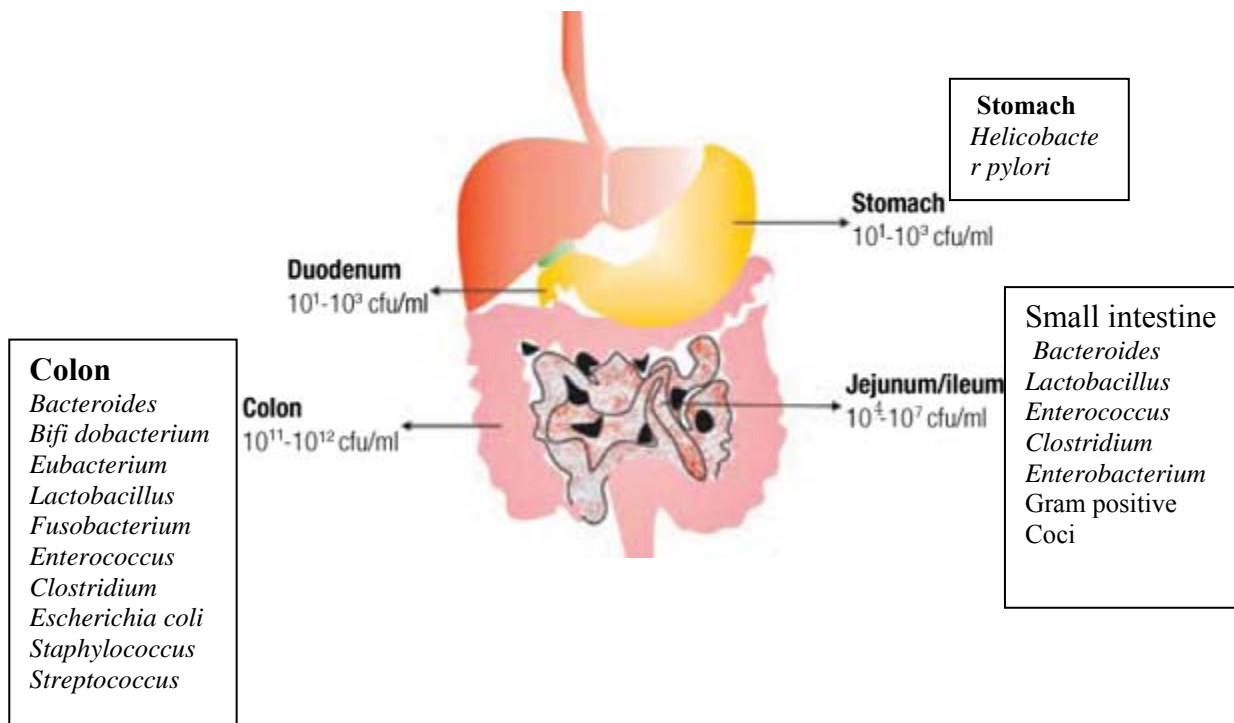


Figure 2.5 Distribution of microflora in gut (adapt from Tsukumo et al, 2009)

2. 8. 2 Gut Microbiota and Energy Metabolism

Type 2 diabetes and Obesity are two metabolic diseases that are becoming epidemics not only in developed western countries but also in developing countries. Obesity per se is shown to be a causal factor for type 2 diabetes that is evidenced by interventional weight reduction and decrease of diabetes incidence in multiple studies (Laville et al, 2009; Horton et al, 2010; Fujioka et al, 2010). Numerous studies have shed light on the combination of variable environmental and genetic factors as causal factors

in the development of these two metabolic diseases. Excessive energy intakes as well as decreases of physical activity are two common external factors. However, within families, some individuals are more prone to have diet-induced weight gain and hyperglycemia, even though they were exposed to similar nutritional circumstances (Turnbaugh et al, 2009; Hill et al, 1998; Christakis et al, 2007; Tappy et al, 2007). Such observations suggested apart from traditional triggers, other novel factors also are involved. Over the last several years, there is growing evidence of gut microbiota as a potential environmental factor in the control of energy homeostasis (Ley et al, 2005; Ley et al, 2006; Turnbaugh et al, 2006; Backhed et al, 2007).

2.8.2.1. Evidence of Association between Gut Microbiota and Metabolic Diseases

A number of studies have revealed the composition of gut microflora changes in obesity and diabetes. Larsen et al reported the proportion of Firmicutes was significantly lower in diabetics compared to the control group and the reduction in *Clostridium* spp was the main reason for the decrease. Although, phyla Bacteroidetes and Proteobacteria were not observed significantly enriched in the diabetic group, the authors found a positive correlation between the ratio of Bacteroidetes to Firmicutes and blood glucose (Larsen et al, 2010). Ley and coworkers analyzed 5,088 bacterial gene sequences and found variation in gut microbiota between obese and lean mice. Obese mice demonstrated a reduction in *Bacteroidetes* at 50% and proportional elevation in *Firmicutes* compared with lean mice (Ley et al, 2005). Similar findings observed in human, obese people tend to have fewer *Bacteroidetes* and more *Firmicutes* than lean people. Moreover, loss of weight after being on a hypocaloric diet resulted in the reversal of microflora in obese to that observed in lean (Ley et al, 2006; Turnbaugh et al, 2008). Interestingly, it was the number of Bacteroidetes not energy intake that correlated with weight loss (Nadal et al,

2008). However, not all studies supported the ‘high Firmicutes/low Bacteroidetes’ hypothesis (Zhang et al, 2009; Duncan et al, 2008). Duncan et al reported no difference in Firmicutes to Bacteroidetes ratio between obese and lean subjects, neither significant relationships existing between the ratio and BMI. Schwartz and co-workers presented similar results. They suggested that the amount of SCFA produced might be important rather than the ratio of bacteria (Schwartz et al, 2010).

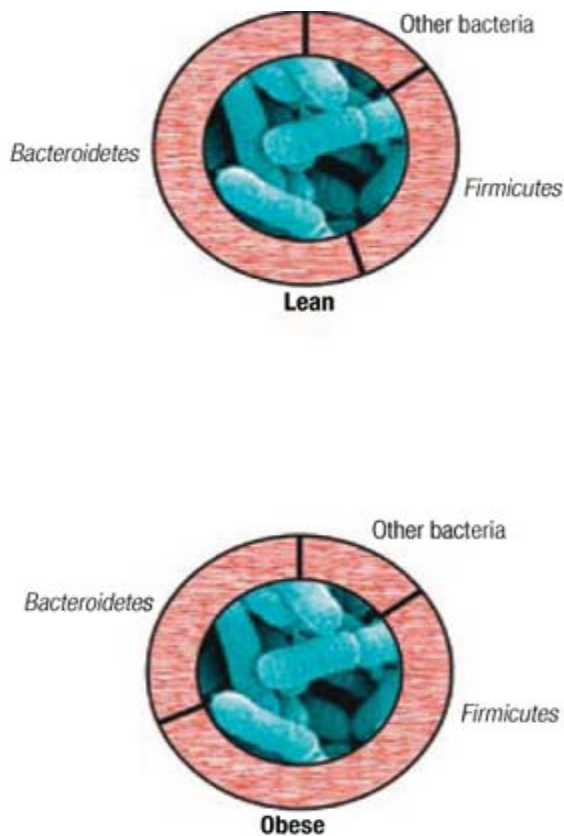


Figure 2.6 Proportion of Firmicutes and Bacteroidetes in lean and obese (Tsukumo et al, 2009)

Studies of germ-free mice and microbiota transplantation further demonstrated how gut microbiota affects the energy metabolism. Gordon’s group found that germ-free mice had higher energy intake but exhibited 40% lower body weight, 42% less total body fat and a 47% reduction of gonadal fat compared with conventional mice with the same age and genetic background. Interestingly, after colonized with gut microflora from

conventional mice, the germ-free mice gained 60% in body fat along with increased insulin resistance within 2 weeks (Backhed et al, 2004). To test whether microflora profile is critical in the fat gain, the germ-free mice were transplanted with gut microflora from ob/ob mice and lean mice, respectively. They showed that the microbiota from ob/ob mice resulted in a greater fat gain than that from lean mice. They also confirmed that ob/ob microbiota recipients had higher relative abundance of firmicute than lean microbiota recipients (Tumbaugh et al, 2006).

2.8.2.2. Mechanisms underlying the Association between Gut Microbiota and Metabolic Diseases

- Energy Harvesting Theory

One of the biological functions of microflora is to digest otherwise indigestible dietary compound (polysaccharides), thereby they provide extra energy to the host. The different composition of microflora observed in lean and obese provoked a hypothesis that the gut bacteria from obese may be more efficient in extracting additional energy from the diet. Backhed and colleagues showed that the microbiota of obese mice had more gene encoding enzymes for breaking down polysaccharides and underwent more fermentation. There were fewer calories left in the feces of obese mice (Backhed et al, 2004). However, it still remains unclear if such a small amount of additional energy extraction could lead to a sufficient weight gain within a short period of time. Besides, non-digestible fiber has been reported to decrease body weight, reduce body fat and improve diabetes (Cani et al, 2006; Shen et al, 2009). Therefore, this observation is not in accordance with the hypothesis of “increased energy harvesting by bacteria” contributing to obesity.

- Induction of Low Grade Inflammation

Obesity and type 2 diabetes are characterized by a low grade inflammation

(Wallen et al, 2005). A recent study showed toll like receptor 4 was involved in the inflammation response triggered by fatty acid following a high fat diet. A high fat diet failed to induce inflammation in toll like receptor 4 knockout mice (Shi et al, 2006). Cani and colleagues found toll like receptor 4 was a coreceptor for lipopolysaccharides (LPS) and LPS was a potent inducer of inflammation derived from breaking down of gram negative bacteria in gut (Cani et al, 2007b). Therefore, they proposed that LPS is a gut microbiota related triggering factor for inflammation. They demonstrated that continuous low rate infusion of LPS caused weight gain and insulin resistance. The CD14 knockout mice, lacking the LPS receptor, did not exhibit the same changes (Cani et al, 2007a). CD14, is a glycosylphosphatidylinositol (GPI)-anchored protein expressed by macrophages (MΦ) and neutrophils, involved in production of proinflammatory cytokines in response to bacterial lysate or purified agonists such as LPS (Sahay et al, 2009). They also reported that a high fat diet led to a reduction of bifidobacteria, an increase of gram negative to gram positive ratio which was associated with a significant elevation of LPS in plasma, blunted insulin sensitivity and diabetes (Cani et al, 2007b). LPS was found higher in type 2 diabetes patients than age matched non-diabetics (Creely et al, 2007). Several studies have indicated that modulation of the gut bacterial population could be effective in improving glycemic control and insulin sensitivity. Using gram negative targeted antibiotics such as norfloxacin and ampicillin improved whole body glucose tolerance and reduced hepatic steatosis/ fatty liver diseases through modification of gut microbiota (Membrez et al, 2008). Dietary intervention with preboitics increased Bifidobacteria which was negatively correlated with LPS concentration and positively correlated with improved glucose tolerance and glucose stimulated insulin secretion in high fat induced obese mice (Cani et al, 2007c).

- Modulation of Energy Homeostasis through Regulating Host Genes

Studies of germ-free mice have indicated the addition of microbiota prompted the host to produce glucose and triacylglycerol in the liver. Fasting-induced adipose factor (FIAF) was a key modulator involved in this fat storage. FIAF can inhibit lipoprotein lipase activity, therefore, when it is suppressed by gut microbiota, the activated lipoprotein lipase promotes release of fatty acids and triacylglycerol which are taken up by adipose tissue (Backhed et al, 2004). In addition, Backhed and colleagues observed that the fatty acid metabolism was increased in germ-free mice. They demonstrated two possible mechanisms that 1) elevated FIAF stimulated the synthesis of peroxisome proliferator activated receptor gamma coactivator which up-regulates expression of gene encoding enzymes in fatty acid oxidation; 2) increased level of AMP-activated protein kinase activity which would promote fatty acid oxidation (Backhed et al, 2007). Modulation of gut microbiota via specific dietary fibers and subsequent upregulation of gut peptide gene expression as well as elevation in plasma is another mechanism proposed (Zhou et al, 2006; Zhou et al, 2009; Cani et al, 2006). Gut hormone such as GLP-1 and PYY are well documented in favor of weight loss and glycemia control (Drab.2010; Horton et al, 2010; Gautier et al, 2008).

CHAPTER 3

DIETARY RESISTANT STARCH IMPROVES MATERNAL GLYCEMIC CONTROL IN GOTO-KAKIZAKI RAT

3.1 Introduction

With the explosion of diabetes reaching epidemic proportion, maternal hyperglycemia is becoming a health threat to pregnant women worldwide, which consists of diabetic pregnant women and those diagnosed with Gestational Diabetes Mellitus (GDM). Maternal hyperglycemia increases risks in labor for mothers. Studies also showed by contributing to the abnormal fetal environment, maternal hyperglycemia may predispose offspring to develop metabolic disorders (Hillier et al, 2007; Boney et al, 2005). Approaches for treatment of maternal hyperglycemia are limited due to safety concerns for the fetus.

RS are dietary carbohydrates that resist digestion in the small intestine and reach the large intestine where they are fermented by bacteria to produce short chain fatty acids. We have shown that feeding resistant starch decreases body fat accumulation in rodents, increases gut GLP-1 gene expression and plasma level, and improves glucose tolerance in STZ-induced diabetic mice (Keenan et al, 2006; Zhou et al, 2006; Zhou et al, 2008). However, the effects of dietary resistant starch on maternal hyperglycemia remain unknown. In this study, therefore, we measured the impact of RS feeding on improving glycemic control in pregnant type 2 diabetes rats-GK rats. Additionally, we focused on identification of the primary factors underlying the mechanism involved.

3.2 Methods and Materials

3.2.1 Animals and Diet

Twenty female Goto-Kakizaki rats aged 5 weeks and weighing 80~100g along with 10 age matched female Wistar rats at the beginning of the study, were obtained from

Charles River (Wilmington, MA). They were housed individually in hanging wire-mesh cages in a temperature-controlled room (22 ± 1 °C) on a 12 h/12 h light/dark cycle with the light on at 7am. Rats were acclimated for 1 week to a powdered diet and to the cages. Water and assigned diet were available *ad libitum* during the experiment except as noted. The protocols were approved by the Pennington Biomedical Research Institutional Animal Care and Use Committee.

The composition of the two experimental diets used in this study is listed in Table 3.1. The resistant starch (RS) diet contained 30% (weight/weight) resistant starch (Hi-Maize® cornstarch; National Starch & Chemical Co., Bridgewater, NJ). The equal energy density control (EC) diet had 100% amylopectin cornstarch (Amioca®; National Starch and Chemical Co.) as the carbohydrate source and equal energy density as the RS diet (3.3kcal/g) by using non-fermentable cellulose (Dyets, Bethlehem, PA) to dilute the energy density.

3.2.2 Experimental Design

After one week of acclimation, GK rats were randomly grouped and divided into two diet treatment groups, resistant starch and energy control, stratified by their weight. Wistar rats served as a genetic control and were fed with energy control diet. The three groups of rats were fed their assigned diets throughout the experiment. Food intake and body weight were measured three times per week. After they were on the diets for 70 days, the animals were mated with male Wistar rats by caging two opposite gender rats together. Pregnancy was confirmed by the presence of a vaginal plug. The rats were sacrificed via decapitation when pups were weaned. Different fat pads (ovarian fat, perirenal fat, and remaining fat in the abdominal area, defined as abdominal fat) were

Table 3.1. Experimental Diet Composition

Ingredients	Control		RS	
	grams	kcal	grams	kcal
100% amylopectin	424.5	1485.8	0	0
High amylose starch				
60% amylose/ 40% amylopectin	0	0	530.7	1486
Sucrose	100	400	100	400
Casein	200	716	200	716
Soybean oil	70	591.5	70	591.5
Cellulose	156.2	0	50	0
Mineral mix	35	30.8	35	30.8
Vitamin mix	10	38.7	10	38.7
Choline chloride	1.3	0	1.3	0
L-cystine	3.0	12	3.0	12
	1000 g/kg	3.3kcal/g	1000 g/kg	3.3kcal /g

removed and weighed. Total body fat used for body fat calculation was the sum of ovarian fat, perirenal fat, and abdominal fat. The gastrointestinal (GI) tract was removed weighed after removal of mesenteric fat. Disemboweled weight was calculated by subtracting GI weight from body weight. The weight of cecal contents was determined by subtraction of empty cecum weight from full cecum weight.

3.2.3 Plasma Assays

Blood was collected and centrifuged at 4000 X g for 20 minutes to extract serum. Serum GLP-1 was measured by radioimmunoassay with RIA kits from Linco Research Inc. (St. Louis, MO). To make the standard curve for GLP-1, 100 ul of the seven standards (10-1000pM) was mixed with 100ul GLP-1 antibody and 400ul assay buffer in tubes to incubate overnight at 4 °C. On the second day, 100 ul ¹²⁵I-Rat GLP-1 was added into the mixture and incubated overnight at 4 °C. On day three, 10 ul Rabbit Carrier and 1.0 cold precipitating reagent were pipetted into the tubes in turn. After following incubation and centrifugation, supernatant was decanted from the tubes and radiation counts were determined with a gamma counter. The counts were regressed on the GLP-1 standard concentration to obtain the standard curve. For the sample measurement, the same procedure was performed. The GLP-1 concentration in each sample was calculated using the standard curve, expressed in pM. Serum insulin was measured using rat ELISA kits from Crystal Chem Inc (Downers Grove, Illinois).

3.2.4 Immunohistochemical Staining and Morphometry

Pancreases were removed from decapitated rats, weighed and fixed in 10% buffered neutral formalin for at least 48hours and embedded in paraffin. Each pancreatic block was sectioned serially at 5um throughout the length to avoid any bias. Twelve

pancreases were examined as total for three groups. Adjacent sections were obtained one in every 20 sections through the specimen and immunostained for insulin by immunofluorescent method (Movassat et al, 1997). The sections were deparaffinized and rehydrated in xylene substitute and ethanol. Blocking buffer was composed of 10% normal goat serum, 0.3% triton X-100 and phosphate-buffered saline. The primary anti-insulin serum was purchased from Invitrogen Corporation (CA, USA). It was a guinea pig anti-porcine insulin serum with 1:200 dilutions in PBS. The second antibody was Alexa Fluor 488 goat anti-guinea pig immunoglobulin at a concentration of 5 ug/ml diluted in PBS, also from aforementioned company. The sections were visualized using fluorescence microscopy. Counterstaining with haematoxylin was performed on each section to facilitate nuclear identification. Quantitative evaluation was performed using nanozoomer digital pathology software (Hamamatsu, Japan). The areas occupied by insulin positive cells as well as the area of the total pancreatic cells were analyzed in each section. The average percent of beta cells to the total pancreatic area of each section was calculated as relative beta cell density.

3.2.5 Cecal Butyrate Producing Bacterial mRNA Expressions

- DNA Extraction

DNA was extracted using a QIAamp DNA Stool Mini kit (QIAGEN, Valencia, CA) using the manufacturer's instructions with slight modifications shown below. An amount of 180-220 mg cecal contents was weighted in a 2ml microcentrifuge tube and placed on ice. Buffer ASL, 1.4ml, was added to each sample and vortexed continuously for 1 min. The following steps were performed at room temperature. The suspension was heated for 5min at 95 °C, and then frozen in liquid nitrogen. The heat-freeze cycle was

repeated three times. After being vortexed for 15s and centrifuged at full speed for 1 min to pellet particles, 1.2 ml of supernatant was removed into a new 2ml microcentrifuge tube. One inhibitEX tablet was put into each sample and vortexed immediately and continuously for 1 min or until the tablet was completely suspended. The suspension was then incubated for 1 min at room temperature to allow the inhibitor to absorb the inhibitEX matrix. Centrifuging at full speed for 3 min before and after the supernatant was removed into a new 1.5 microcentrifuge tube. Proteinase K, 15 ul, was pipeted into a new microcentrifuge tube with 200 ul supernatant. After adding 200 ul buffer AL, the tube was incubated at 70 °C for 10 min. then the solution in the tube was mixed with 200ul of 100% ethanol, and the lysate was put into a spin column. The DNA was washed with 500ul buffer AW1 and 500ul buffer AW2 respectively; a one-minute spin at full speed was applied after a wash and a 3-minute spin for the last wash. Then the column was moved to a 1.5-ml collection tube, and 200 ul of buffer AE was added directly onto the fiber matrix. After a 1-minute incubation at room temperature, the cup was spun for 1 min. For DNA quantification, 1.5 ul of sample DNA was used to detect the optical density (OD) at OD260 and OD280 using a nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. DE, USA).

- Quantitative Real-time PCR

The gene transcription for butyrate producing bacteria was determined using the SYBR® Green method of quantitative real-time PCR (qRT-PCR) assay, and results were expressed as a relative fold change of control. The sequences of the primers for targeted bacterial groups are listed in Table 3.2. Real time RT-PCR reaction mixture was 10 ul of total volume, including 3 µl of DNA sample, 5µl of 2X SYBR Green Master Mix

(Applied Bio systems, Foster City, CA,USA), 0.5 µl of reverse/forward primers at 10 µM, 0.5 µl of bovine serum albumin (BSA) (final concentration 2.5 mg/ml), and 0.5 µl of nuclease free water. All reactions were performed in sterile MicroAmp® optical 384-well reaction plates (Applied Biosystems, Foster City, CA). The reaction condition is 50°C for 2 min, 95°C for 10 min for one cycle, then 40 cycles of 95°C for 15 s, 60°C for 1 min, then 78°C for 30 s. The dissociation step was included to verify specificity through analyzing the melting curve of the amplified product.

3.2.6 Measurement of UCP-1 mRNA Expression

RNA was extracted from brown adipose tissue using Trizol from Sigma (St. Louis, MO). The gene transcription for UCP-1 was determined using real-time reverse transcriptase polymerase chain reaction, and results were expressed as a ratio to the expression of the constitutive gene cyclophilin. The sequence of the primers and probe for rat cyclophilin is listed in Table 3.2. The probe and primers for UCP-1 (assay identification no. Rn00562126_m1) were purchased from Applied Biosystems (Foster City, CA). Real time RT-PCR reaction mixture was 10 µl of total volume, including 9ng of sample RNA, 1 µl of 10 X Taqman buffer, 5.5mM MgCl₂, dATP, dCTP, dUTP and dGTP each 0.3 mM, 500 nM forward primers, 500 nM reverse primers, 200 nM Taqman probes, 7.5 U RNase inhibitor, 5 U MuLV reverse transcriptase, 0.3 U AmpliTaq Gold DNA polymerase and RNase-free H₂O. Each sample was tested in duplicate. The one-step real-time reverse transcriptase polymerase chain reaction condition is 48 °C for 30 min, 95°C for 10 min for one cycle, 95 °C for 15 sec and 60 °C for 1 min for 40 cycles.

Table 3.2 The sequences of primers for real time RT-PCR. F: forward primer, R: reverse primer, P: probe.

Gene	Sequence
16S universal primers	F - TGSTGCAYGGYYGTCGTCA R -ACGTCRTCCMCNCCTTCCTC
Bifidobacterium spp	F –GGGTGGTAATGCCGGATG R- TAAGCCATGGACTTTCACACC
Bacteroidetes	F -GAA GGT CCC CCA CAT TG R- CAA TCG GAG TTC TTC GTG
Lactobacillus spp	F -TGG ATG CCT TGG CAC TAG GA R- AAA TCT CCG GAT CAA AGC TTA CTT AT
Clostridial cluster IV	F- TTA CTG GGT GTA AAG GG R- TAG AGT GCT CTT GCG TA
Clostridium cluster XIV	F- AAA TGA CGG TAC CTG ACT AA R- CTT TGA GTT TCA TTC TTG CGA
Cyclophilin	F- 5'CCCACCGTGTTCTTCGACAT3' R- 5'TGCAAACAGCTCGAAGCAGA 3' P- 5'CAAGGGCTCGCCATCAGCCG 3'

3.2.6 Oral Glucose Tolerance Test

The oral glucose tolerance test was performed in female rats on the 16th day of gestation. After an overnight fast, a blood sample was collected for insulin measurement. Blood glucose was measured prior and 30, 60, 120 minutes after the administration of glucose (2.0 g/kg body weight) with a glucometer (Abbott Laboratories, North Chicago, IL). HOMA-IR was calculated to indicate insulin sensitivity.

3.2.7 Pancreatic Insulin Content

Approximately 150mg of pancreas was weighed and placed into 2ml Acid-Ethanol solution (75% ethanol, 1.5% HCL 12N, 23.5% distilled water). After an overnight incubation at -20 °C, the tissue was homogenized at 4 °C and then underwent another overnight incubation at -20°C. The diluted supernatant was used to determine insulin content by ELISA (Crystal Chem Inc., Downers Grove, Illinois, USA).

3.2.8 Cecal Contents pH and Short Chain Fatty Acid Analysis

Cecal contents (0.5 gram) was weighed and put into a plastic centrifuge tube with 4.5 ml of distilled water. The sample was mixed thoroughly by vortex and pH was measured using a pH meter. The sample was centrifuged at 4°C at 8000 RPM for at least 10 min and filtered through a Millipore syringe filter. The supernatant was transferred to a vial and the vial stored at 0°C until short fatty acids were quantitated by gas chromatography.

3.2.9 Statistical Analysis

Data are presented as means \pm SEM. Statistical analyses were performed using the Statistical Analysis System (SAS 9.1). One way ANOVA and student t test were used to

examine the influence of treatment on all measurements. Subgroup means were compared by Tukey's method.

3.3 Results

3.3.1 Fat Pads Weights

Compared with GK rats fed the control diet, dietary resistant starch significantly decreased fat/disemboweled weight ($p<0.05$) (Figures 3.1).

3.3.2 Insulin Sensitivity and Fasting Glucose Levels

Dietary resistant starch improved insulin sensitivity in pregnant GK rats as indicated by HOMA-IR. Also resistant starch fed pregnant GK rats had lower fasting glucose and fasting serum insulin concentrations compared with EC fed GK rats ($p<0.05$) (Figures 3.2). However there was no difference found in Δ AUC between these two groups.

3.3.1 Immunohistochemistry and Pancreatic Insulin Content

There were fewer islets in the GK groups than in normal Wistar rats; the GK-EC rats had the least. RS-GK rats had more pancreatic insulin than EC fed GK rats ($p<0.05$). The beta cell relative densities were $0.758 \pm 0.064\%$ in Wistar rats, $0.301 \pm 0.024\%$ in GK-EC rats, and $0.56 \pm 0.037\%$ in GK-RS rat, respectively ($p<0.05$). In the GK-EC rats, the large islets displayed disrupted configuration, irregular capsules and cells of unevenly being stained with the anti-insulin sera. In contrast, islets in Wistar rats were round in shape, clearly boundary defined, and homogeneously stained beta cells (Figure 3.3).

3.3.4 Cecal Content pH and Short Chain Fatty Acids

The pH values for both cecal contents and feces were lower in RS fed pregnant GK rats, whereas the weights of full GI and cecal content were higher in RS fed pregnant GK

rats than EC fed GK rats ($p<0.05$) (Figure 3.4). Short chain fatty acid concentrations including acetate, butyrate and propionate, were elevated in cecal contents of pregnant GK rats fed RS ($p<0.05$) (Figure 3.7).

3.3.5 Microflora Expressions in Cecum

The microflora engaged in converting resistant starch to butyrate are *Bacteroides* spp, *Bifidobacterium* spp, *Lactobacillus* spp, Clostridial cluster IV and Clostridial cluster XIV (Bird et al, 2000; Xu et al, 2003a; Louis et al, 2007b; Louis et al, 2007a; Sato et al, 2008). The population of bacteria involved in butyrate production in the cecum was increased in RS fed pregnant GK rats. *Bacteroides*, *Bifidobacterium*, *Lactobacillus* and Clostridial cluster IV population were increased compared with those in EC fed GK rats ($p<0.05$) (Figure 3.5, 3.6). No significant difference was observed in Clostridial cluster XIV.

3.3.6 Serum GLP-1 Concentration and UCP-1 Gene Expression in BAT

The total serum GLP-1 concentration was increased in GK rats fed with RS, compared with GK rats fed with EC ($p<0.05$) (Figures 3.8). There was a trend of increased mRNA expression of UCP-1 in brown adipose tissue of rats fed with RS ($p=0.08$).

3.3.7 Food intake and Disemboweled Weight

There were no statistical differences in food intake between control and RS fed rats. This demonstrated no or minimal discomfort with the consumption of resistant starch at the levels fed. Because RS fed rats had significantly heavier GI contents, the disemboweled body weight was used to exclude GI contents from body weight. There

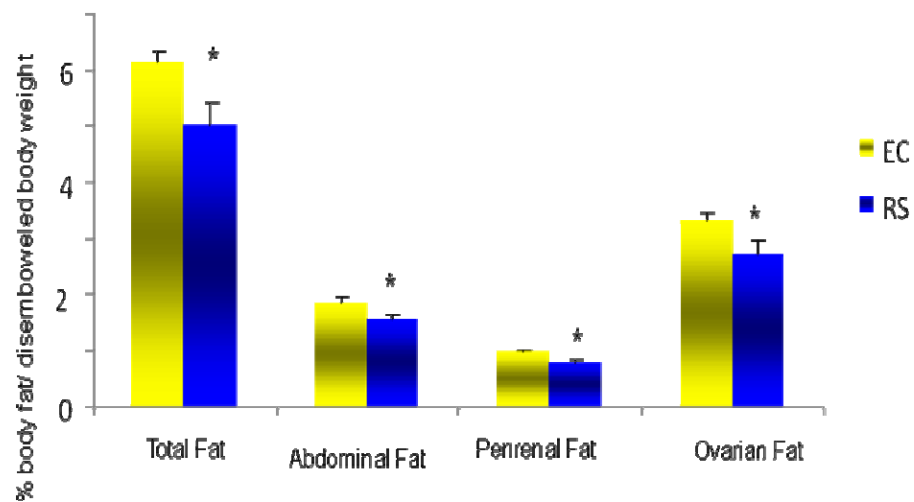


Figure 3.1 Percentages of body fat/ disemboweled body weight in GK rats fed with resistant starch or energy control diet. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC treated GK rats.

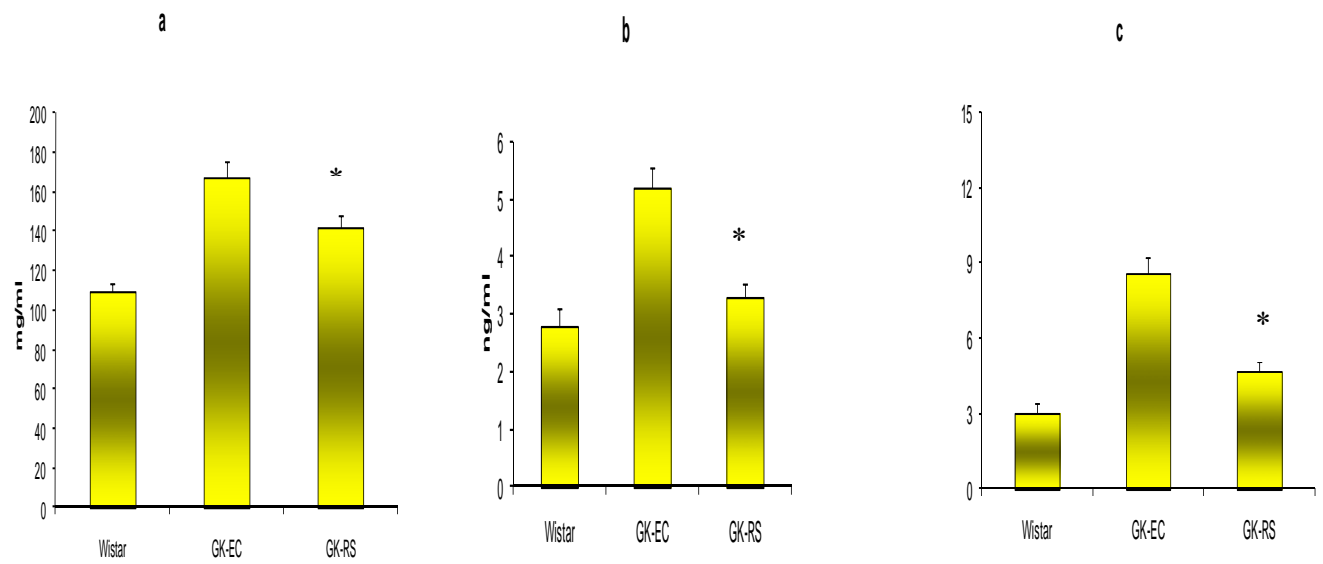


Figure 3.2 Fasting glucose concentrations (a), fasting serum insulin levels (b) and HOMA-IR (c) measured on the 16th day of gestation in GK rats fed with RS or EC diet and Wistar rats fed on EC. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC-GK.

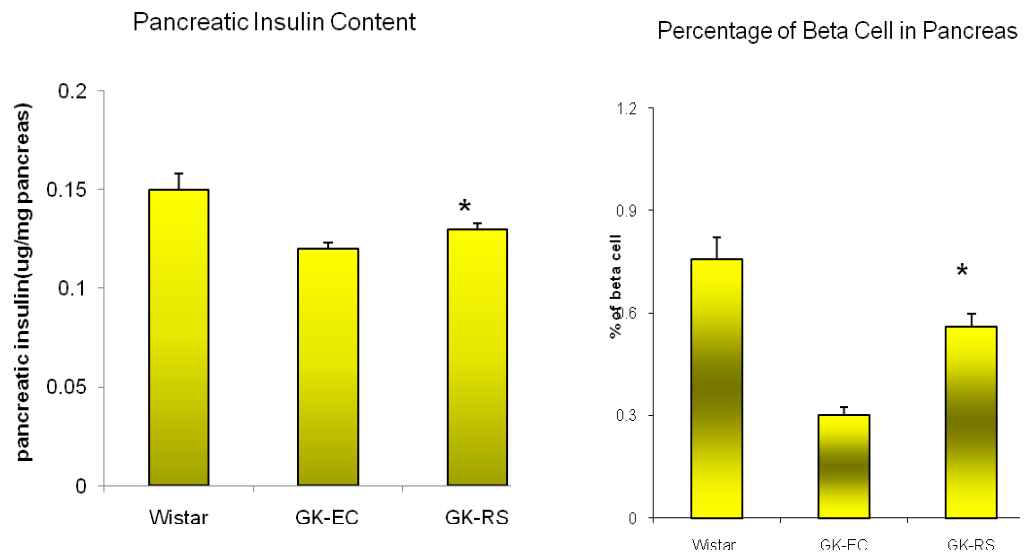
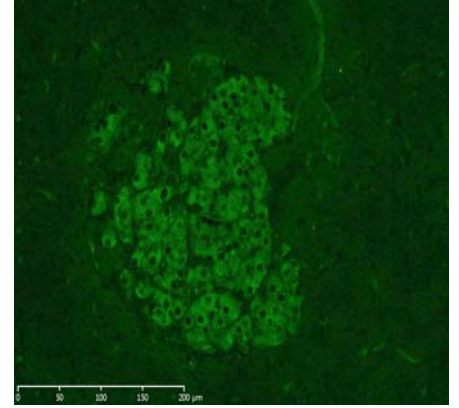
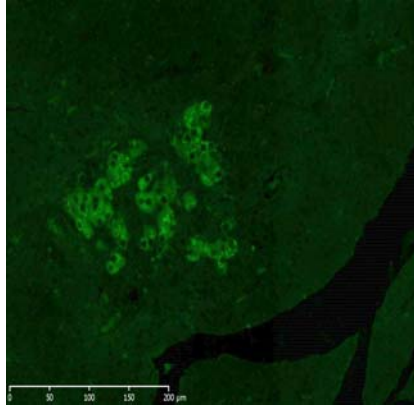
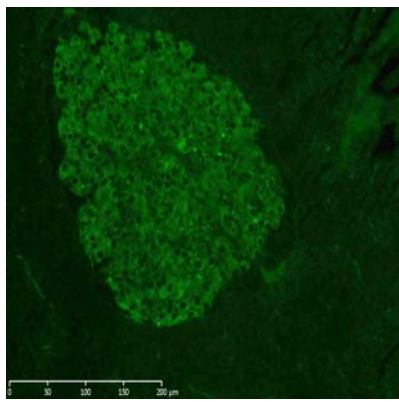


Figure 3.3 Beta cell densities and pancreatic insulin contents in GK rats fed with RS or EC diet and Wistar rats fed on EC diet. Data are mean \pm SEM. * $P < 0.05$ vs. EC-GK.

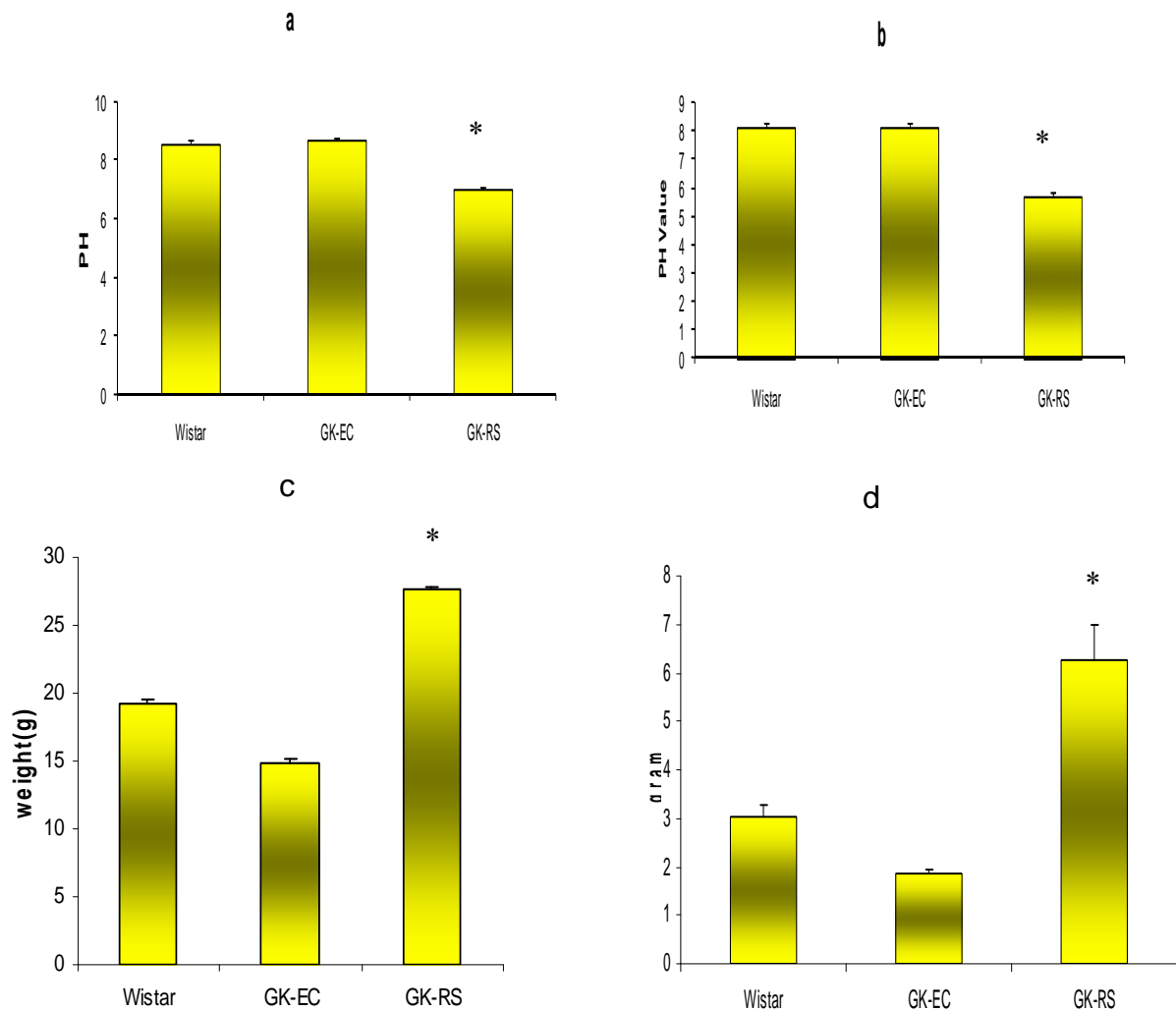


Figure 3.4 Cecal content pH(a), feces pH values (b), full GI weight (c) and cecal content weight (d) in GK rats fed with RS or EC diet and Wistar rats fed on EC diet. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC-GK.

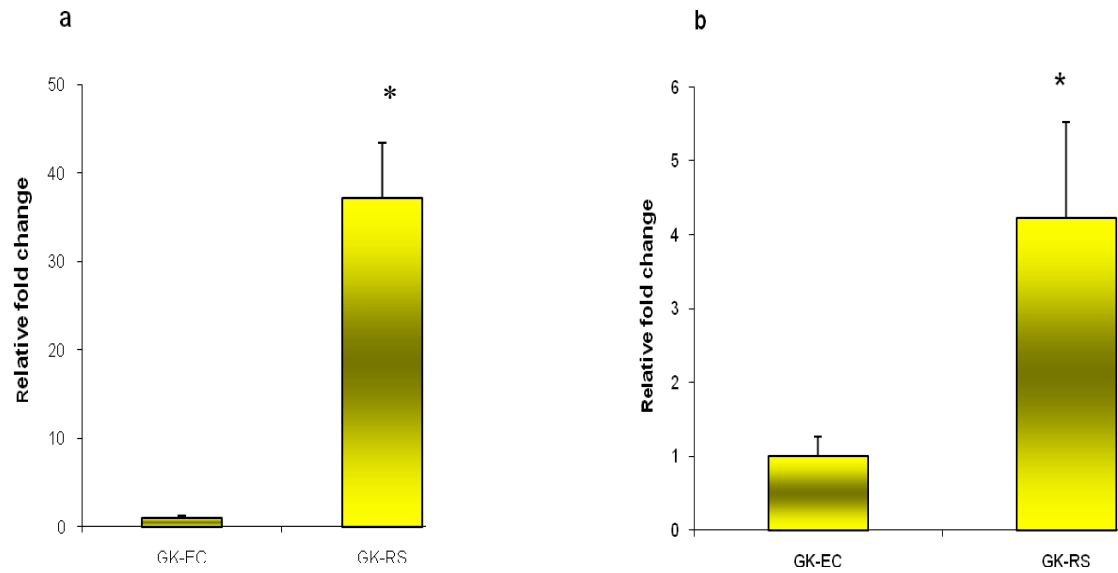


Figure 3.5 Fold changes of *Bacteroides* spp (a) and *Bifidobacterium* spp (b) measured with RT-PCR in GK rats fed with RS or EC diet. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC-GK.

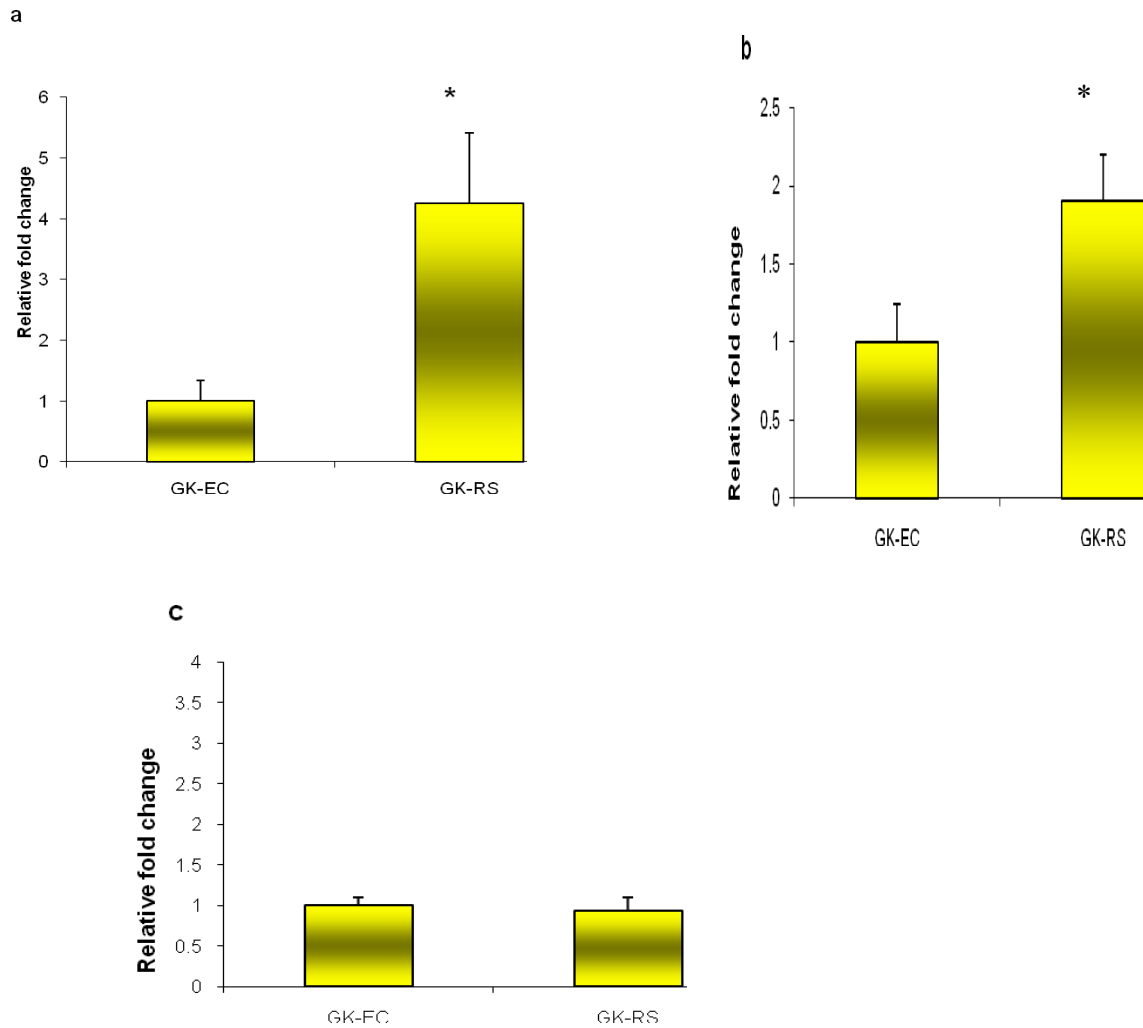


Figure 3.6 Fold changes of *Lactobacillus* spp (a), *Clostridial* cluster IV(b) and *Clostridial* cluster XIV (c) were measured with RT-PCR in GK rats fed with RS or EC diet . Data are mean \pm SEM for group of 10 rats. * P<0.05 vs. EC-GK.

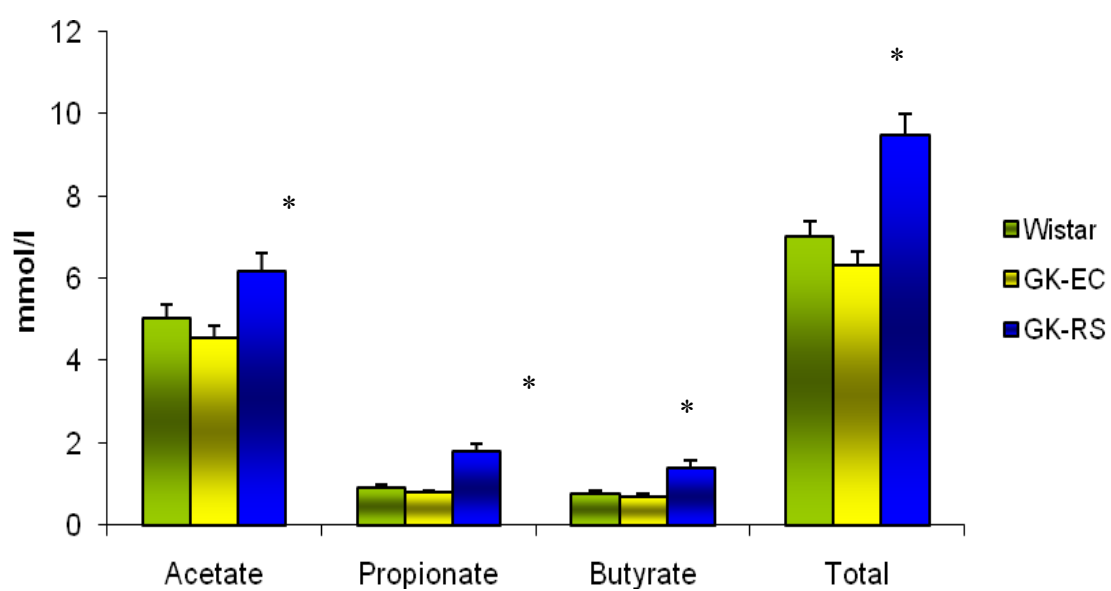


Figure 3.7 Cecum short chain fatty acids concentrations were measured in GK rats fed with RS or EC diet and Wistar rats on EC for 18 weeks. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC- GK.

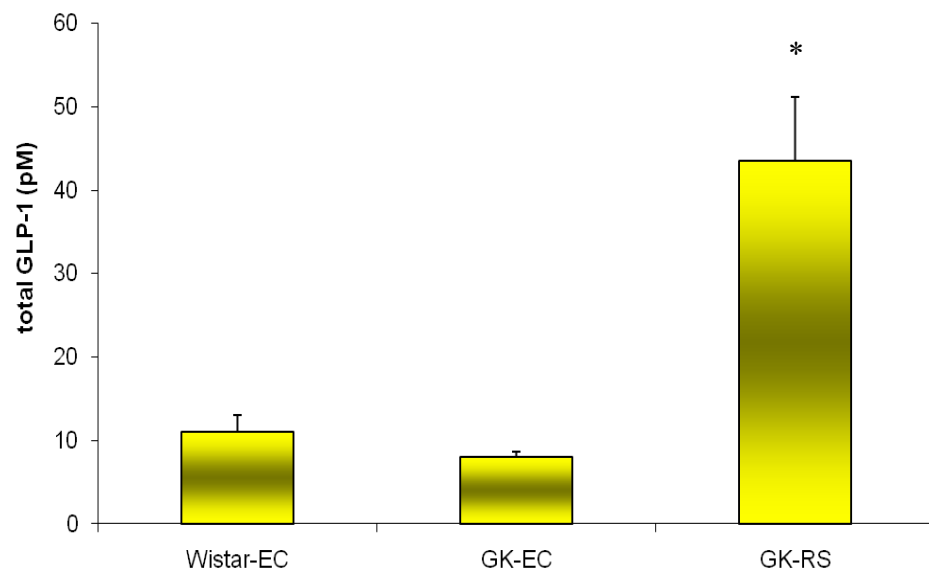


Figure 3.8 Serum total GLP-1 concentrations in GK rats fed with RS or EC diet and Wistar rats on EC diet for 18 weeks. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC- GK.

Table 3.3 Food intakes and disemboweled body weight

Group	Cumulative food intake (g)	Disemboweled body weight (g)
GK-EC	1442.0 ± 36.0	274 ± 5.1
GK-RS	1565.6 ± 52.9	262 ± 6.55

GK-EC: GK rats fed control diet

GK-RS: GK rats fed resistant starch diet

Food intake and disemboweled body weight of GK rats fed with RS or EC diet for 18 weeks. Data are mean \pm SEM for group of 10 rats

was no significant difference for disemboweled body weight between control and RS fed rats (Table 3.3)

3.4 Discussion

In this study, we investigated the effects of dietary resistant starch on improving maternal hyperglycemia. Our results demonstrated that dietary resistant starch increases insulin sensitivity and pancreatic beta cell mass in pregnant GK rats, a non obesity type 2 diabetes model featured with reduced beta cell mass and defective insulin response to glucose (Portha, 2005; Movassat et al, 1997; Movassat et al, 2007; Movassat et al, 1995). Specifically, we measured fat pad changes, fasting insulin and glucose concentration, pancreatic insulin content and beta cell density in the pregnant GK rats in the context of resistant starch feeding. To our knowledge, our findings provide the first direct evidence that dietary resistant starch alters pancreatic beta cell density in GK rats.

Feeding resistant starch to pregnant GK rats significantly increased insulin sensitivity. The result is consistent with the observation that RS fed rats had reduced fat pads. The decreased body fat in RS fed rats is most likely the result of increased energy expenditure. Human studies have shown fatty acid oxidation is significantly increased after consumption of resistant starch (Higgins et al. 2004). Our previous data also suggested dietary resistant starch enhanced energy expenditure in mice (Zhou et al, 2008). It increased the expression of POMC in the arcuate nucleus of the hypothalamus in rats (Shen et al, 2009) which is critical in promoting energy expenditure (Xu et al, 2006), and increased protein expression of adiponectin in white adipose tissue (unpublished data). We found the butyrate levels were elevated in the cecal contents of GK rats with the feeding of resistant starch. Gao and his coworkers reported that the addition of sodium

butyrate in diet led to improved insulin sensitivity, facilitated fatty oxidation and increased energy expenditure in mice; it protected mice from diet induced obesity (Gao et al, 2009). Different from the butyrate in diet, butyrate from fermentation has local effect in the lower gut where butyrate is avidly absorbed by the colonocytes and L-endocrine cells to stimulate GLP-1 expression (Zhou et al, 2006). Some butyrate may reach the liver to inhibit hepatic lypolysis (unpublished data). However, whether the butyrate from fermentation enters systemic blood is still uncertain. Therefore, the effect of the butyrate from fermentation may be indirect through the production of gut hormones. GLP-1 is reported to increase energy expenditure. Higher fasting plasma GLP-1 levels are associated with higher rates of energy expenditure and fat oxidation in human subjects (Osaka et al, 2005). It has also been reported that GLP-1 plays a role in postprandial energy expenditure and that GLP-1 stimulates POMC neurons in the arcuate nucleus via GLP-1 receptors (Ma et al, 2007). We found a trend of increased UCP-1 expression in resistant starch fed GK rats, compared with EC fed GK rats, which is in accordance with what Aziz et al reported. They observed a high amylose starch diet led to higher insulin sensitivity index (QUICKI), and elevated mRNA expression of UCP-1 in diet induced obese rats (Aziz et al, 2009). UCP-1 diverts energy from ATP synthesis to thermogenesis through which increases energy expenditure (Puigserver et al, 1998).

Another major finding is that feeding resistant starch significantly increased pancreatic insulin content and beta cell density in pregnant GK rats. This result raises an interesting question: what causes such changes in resistant starch fed GK rats? Resistant starch potentially has three major effects as a part of the diet: metabolizable energy dilution, a bulking effect, and fermentation to produce short-chain fatty acids and

increase GLP-1 (Keenan et al. 2006; Zhou et al, 2008). In our study, control and resistant starch diets have the same energy density, so the energy dilution effect can be excluded. The bulking effect is the prevention of food intake caused by gastrointestinal distension (Phillips et al. 2000). But our results indicated there was no difference in food intake between resistant starch fed GK rat and EC fed rats. Thus, the hypothesis most likely for the mechanism of increased pancreatic insulin content and beta cell density was narrowed down to the fermentation of resistant starch and the subsequent increases of GLP-1. Elevated plasma GLP-1 was previously observed before in both normal SD and diet induced obese rats fed resistant starch (Shen et al, 2009; Aziz et al, 2009). The GLP-1 increase is consistent over a 24 hour period in SD rats (Zhou et al, 2008). Gut gene expression data from our group also verified that dietary RS dramatically up-regulated the expression of the GLP-1 gene in rat cecal cells compared to rats consuming an energy control diet (Zhou et al, 2006).

In order to clarify the process from dietary resistant starch fermentation associated with increased production of GLP-1 in resistant starch fed pregnant GK rats, we investigated several important steps in fermentation. Firstly, we measured cecal and fecal pH values, cecal content weight, and found the existence of fermentation demonstrated by the decrease of pH values as well as increased cecal content weight in resistant starch fed GK rats. Secondly, augmented butyrate producing bacterial population was confirmed except for Clostridial cluster XIV which might be due to its absolute reduction in type 2 diabetes (Larsen et al, 2010). Third, short chain fatty acids including acetate, butyrate and propionate were elevated in resistant starch fed GK rats.

Finally, an increase of plasma GLP-1 was identified. Our previous study has indicated butyrate promoted GLP-1 expression in vitro (Zhou et al, 2006).

The action of GLP-1, as a potent incretin, includes stimulating proinsulin gene expression (Drucker et al, 1987), inhibiting glucagon secretion (Nauck et al, 2002). It also mediates glucose-dependent insulin secretion via their receptors expressed on beta cell (Drucker, 2006), inhibits gastric acid secretion and delays gastric emptying (Baggio et al, 2004), as well as promotes an increase in pancreatic β -cell mass through enhancing beta cell proliferation and inhibiting apoptosis (Stoffers et al, 2003; Wang et al, 2002).

GLP-1 was shown to be able to delay the onset of type 2 diabetes and improve pancreatic insulin content and total beta-cell mass in GK rats when applied postnatally for 5 days (Tourrel et al, 2002). This hormone was also reported to reduce apoptosis in human islets (Farilla et al, 2003). A GLP-1 receptor agonist demonstrated similar effects. Exendin-4 not only improved glucose tolerance in diabetic rats via expansion of beta cell volume (Xu et al, 1999), but also prevented the development of diabetes in rats exposed to intrauterine growth retardation (Stoffers et al, 2003). Further studies are needed for a conclusive determination for the role of increased GLP-1 in dietary resistant starch induced improvement on glycemic control in pregnant GK rats.

In conclusion, dietary resistant starch improved insulin sensitivity, pancreatic insulin content and beta cell density in pregnant GK rats. The increased production of GLP-1 resulting from fermentation of resistant starch was linked to the mechanism of improved glycemic control in pregnant GK rats. Our findings provide further evidence that resistant starch works as a natural agent to treat maternal hyperglycemia.

CHAPTER 4

FEEDING DIETARY RESISTANT STARCH TO GOTO-KAKIZAKI RAT IMPROVES FASTING GLUCOSE IN OFFSPRING

4.1 Introduction

The observation of adult type 2 diabetics having higher incidence of type 2 diabetes in mothers than that on the paternal side suggested the role of abnormal fetal environment in the development of metabolic disorders (Dorner et al, 1976). Over the years, there is a growing body of evidence indicating intrauterine exposure to a hyperglycemic environment increased the risk of diabetes and obesity for offspring later in their life in addition to genetic transmission (Ezekwe et al, 1980; Kasser et al, 1981; Hausman et al, 1982; Pettitt et al, 1991; Waterland et al, 1999; Boney et al, 2005). A study also revealed that glycemic control in GDM pregnancies is an effective way to prevent impaired glucose tolerance in childhood. Even minimal intervention will make a difference (Malcolm et al, 2006).

RS are dietary carbohydrates that resist digestion in the small intestine and reach the large intestine where they are fermented by bacteria to produce short chain fatty acids. Previous work from our lab has shown that feeding resistant starch to pregnant GK rats improved insulin sensitivity and hyperglycemia. In this study, therefore, we investigated the offspring of GK rats that had been fed a RS diet or energy control diet from 6 weeks old throughout adult life and pregnancy. All offspring were weaned on a standard chow diet. Food intake, insulin sensitivity, pancreatic insulin content and beta cell mass of the offspring were determined.

Due to its ability to resist being broken down completely by digestive enzymes, it was reported that dietary resistant starch in the diet was accompanied by gastrointestinal

effects including flatus, abdominal discomfort, and diarrhea, which could exert a negative impact on gestation and fetal growth (Grabitske et al, 2009). Therefore, pup size, the growth curve and fat pad weights were observed to elucidate these possibilities.

4.2 Methods and Materials

4.2.1 Animals and Diet

Twenty female Goto-Kakizaki rats aged 6 weeks from Charles River (Wilmington, MA) were housed individually and fed either the resistant starch diet or energy control diet for 10 weeks before they were mated. Ten age -matched female Wistar rats were fed the energy control diet. All animals (GK-EC, GK-RS, and Wistar-EC) were in a temperature-controlled room (22 ± 1 °C) on a 12 h/12 h light/dark cycle with the light on at 7am with free access to water and assigned diet. The protocols were approved by the Pennington Biomedical Research Institutional Animal Care and Use Committee.

The composition of the two experimental diets used in this study is listed in Table 3.1. The resistant starch (RS) diet contained 30% (weight/weight) resistant starch (Hi-Maize® cornstarch; National Starch & Chemical Co., Bridgewater, NJ). The equal energy density control (EC) diet had 100% amylopectin cornstarch (Amioca®; National Starch and Chemical Co.) as the carbohydrate source and an equal energy density as the RS diet (3.3kcal/g) by using non-fermentable cellulose (Dyets, Bethlehem, PA) to dilute the energy density.

After rats in these three groups delivered, their pups were weighed and litter size reduced to 6 pups. Ten pups from each group were randomly chosen and raised to 8 weeks old on standard chow diet (#5001, Dietlab, USA). Food intake and body weight were measured three times a week. Upon being decapitated, different fat pads

(epididymal fat, perirenal fat, and abdominal fat) were removed and weighed. Total abdominal body fat used for body fat calculation was the sum of epididymal fat, perirenal fat, and abdominal fat. The gastrointestinal (GI) tract was removed and weighed after removal of mesenteric fat. Disemboweled weight was calculated by subtracting GI weight from body weight. The weight of the cecal contents was determined by subtraction of empty cecum weight from full cecum weight.

4.2.2 Plasma Assays

Blood was collected and centrifuged at 4000 X g for 20 minutes to extract serum. Serum insulin was measured using rat ELISA kits from Crystal Chem Inc (Downers Grove, Illinois).

4.2.3 Immunohistochemical Staining and Morphometry

Pancreases were removed from decapitated rats, weighed and fixed in 10% buffered neutral formalin for at least 48 hours and embedded in paraffin. Each pancreatic block was sectioned serially at 5 μ m throughout the length to avoid any bias. Twelve pancreases were examined as total for three groups. Adjacent sections were obtained with one in every 20 sections through the specimen and immunostained for insulin by immunofluorescent method (Movassat et al, 1997). The sections were deparaffinized and rehydrated in xylene substitute and ethanol. Blocking buffer was composed of 10% normal goat serum, 0.3% triton X-100 and phosphate-buffered saline. The primary anti-insulin serum was purchased from Invitrogen Corporation (CA, USA). It was a guinea pig anti-porcine insulin serum with a 1:200 dilutions in PBS. The second antibody was Alexa Fluor 488 goat anti-guinea pig immunoglobulin at a concentration of 5 μ g/ml diluted in PBS, also from the aforementioned company. The sections were visualized

using fluorescence microscopy. Counterstaining with haematoxylin was performed on each section to facilitate nuclear identification. Quantitative evaluation was performed using nanozoomer digital pathology software (Hamamatsu, Japan). The area occupied by insulin positive cells as well as the area of the total pancreatic cells was analyzed in each section. The average percent of beta cells to the total pancreatic area of each section was calculated as relative beta cell density.

4.2.4 Oral Glucose Tolerance Test

An oral glucose tolerance test was performed in pups when they were 56 days old. After an overnight fast, a tail vein blood sample was collected for insulin measurement. Blood glucose was measured prior and 120 minutes after the administration of glucose (2.0 g/kg body weight) with a glucometer (Abbott Laboratories, North Chicago, IL). HOMA-IR was calculated to indicate insulin sensitivity.

4.2.5 Pancreatic Insulin Content

Approximately 150mg of pancreas was weighed and placed into 2 ml of Acid-Ethanol solution (75% ethanol, 1.5% HCL 12N, 23.5% distilled water). After an overnight incubation at -20 °C, the tissue was homogenized at 4 °C and then underwent another overnight incubation at -20°C. The diluted supernatant was used to determine insulin content by ELISA (Crystal Chem Inc., Downers Grove, Illinois, USA).

4.2.6 Cecal Content pH Measurement

Cecal contents, 0.5 grams, were weighed and put into a plastic centrifuge tube with 4.5 ml of distilled water. The sample was mixed throughout by vortexing and the pH was measured using a pH meter.

4.2.7 Statistical Analysis

Data are presented as means \pm SEM. Statistical analyses were performed using the Statistical Analysis System (SAS 9.1). One way ANOVA and student ttest were used to examine the influence of treatment on all measurements. Subgroup means were compared by Tukey's method.

4.3 Results

4.3.1 Fat Pads Weights

There were no significant differences found in percentages of body fat/ body weight between pups born to RS fed GK rats and EC fed GK rats (Figures 4.1).

4.3.2 Insulin Sensitivity and Fasting Glucose Levels

Pups born to dietary resistant starch fed GK rats had lower fasting glucose compared with offspring from EC fed GK rats ($p < 0.05$) (Figure 4.2). However there was no difference found in fasting serum insulin concentration, 2- hour glucose level and insulin sensitivity (HOMA-IR) between these two groups.

4.3.1 Immunohistochemistry and Pancreatic Insulin Content

Pancreatic insulin content was increased in pups born to resistant starch fed GK rats compared to those born to EC fed GK rats. No significant difference was found in beta cell density between offspring from the two groups (Figure 4.3).

4.3.4 Cecal Content pH

The pH value for cecal contents was not significantly different between pups born to GK rats on the resistant starch or the EC diet. Offspring from resistant starch fed GK rats showed lower weight of cecal contents ($p < 0.05$) (Figure 4.4). As indicated by cecal content weight and pH value, there was no fermentation difference in the cecum between

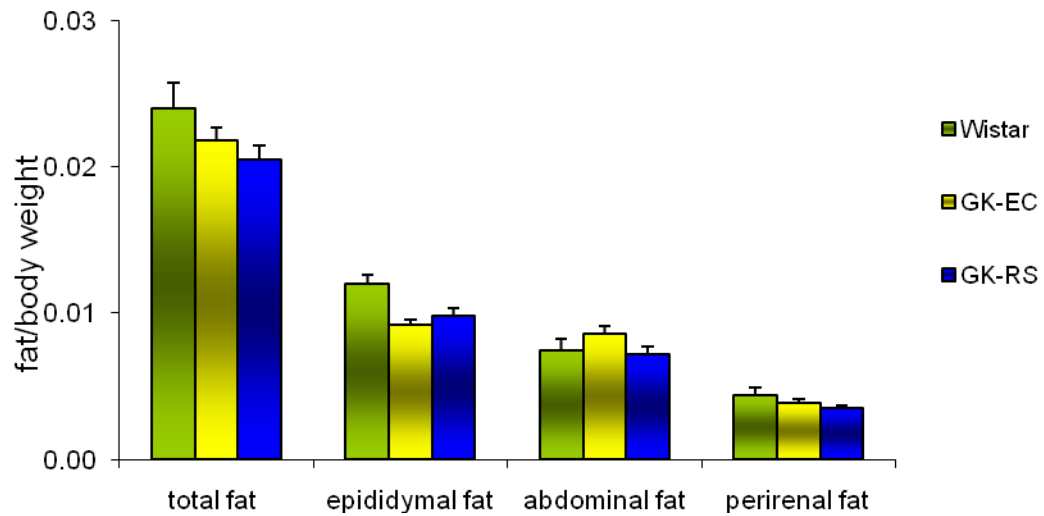


Figure 4.1 Different fat pads (epididymal fat, perirenal fat, and abdominal fat) were removed and weighed from pups from GK-RS, GK-EC and Wistar-EC dams. Total body fat used for body fat calculation was the sum of epididymal fat, perirenal fat, and abdominal fat. Data are mean \pm SEM for group of 10 rats.

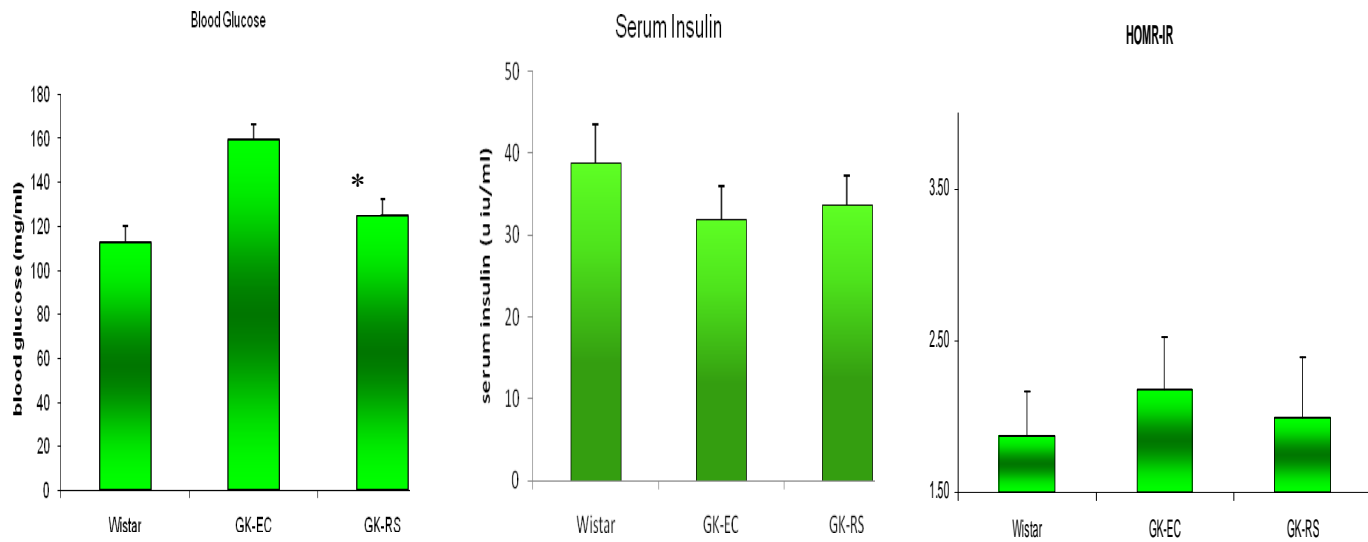


Figure 4.2 Fasting glucose and insulin concentrations as well as HOMA-IR were measured in 8 weeks old pups from GK-RS, GK-EC and Wistar-EC dams. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC-GK.

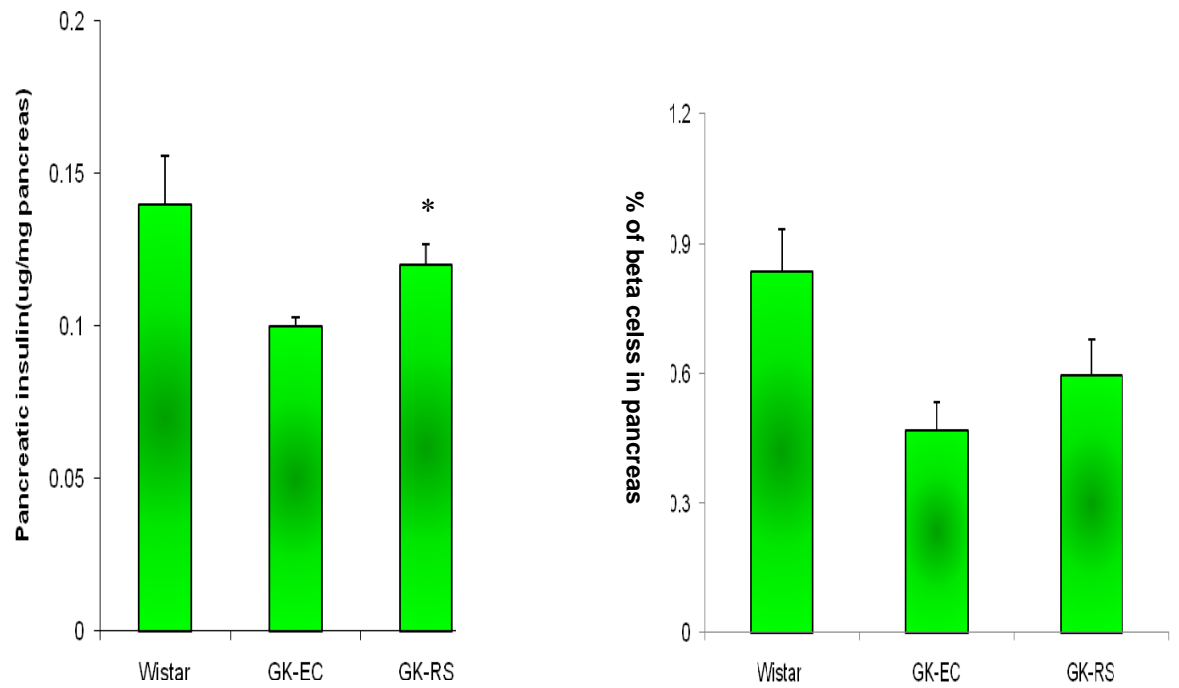


Figure 4.3 Pancreatic insulin contents (left) and Beta cell density (right) were measured in 8 weeks old pups born to GK-RS, GK-EC and Wistar-EC dams. Data are mean \pm SEM. * $P < 0.05$ vs. EC-GK.

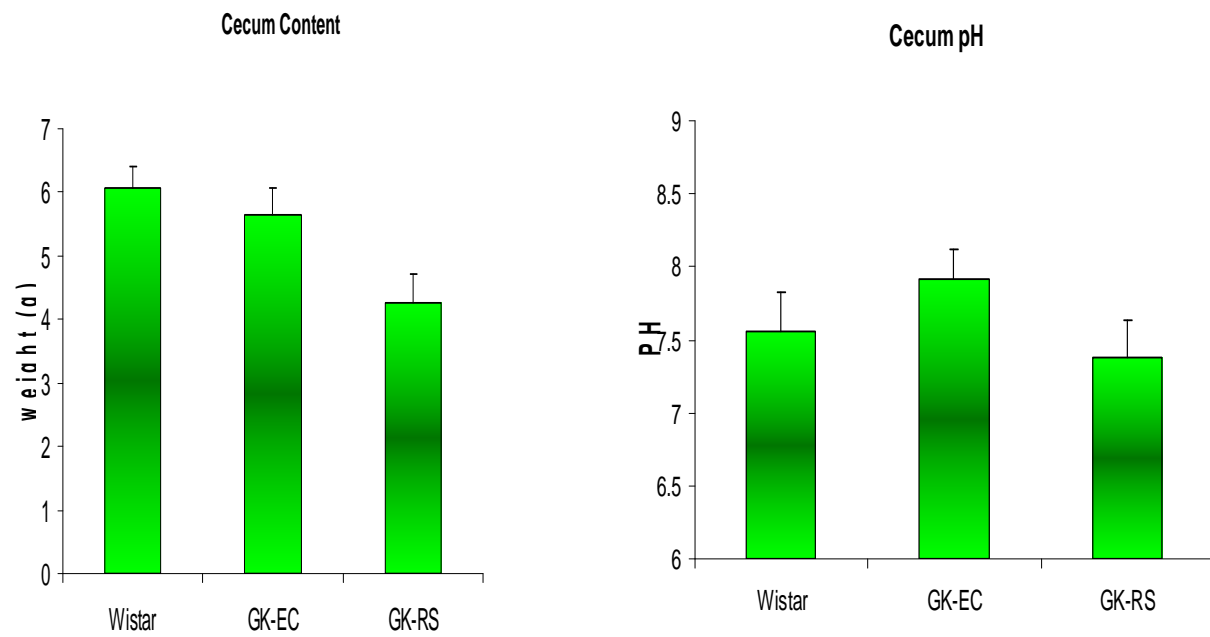


Figure 4.4 Cecum content pH and cecum content weight were measured in 8 weeks old pups born to GK-RS, GK-EC and Wistar-EC dams. Data are mean \pm SEM for group of 10 rats. * $P < 0.05$ vs. EC-GK.

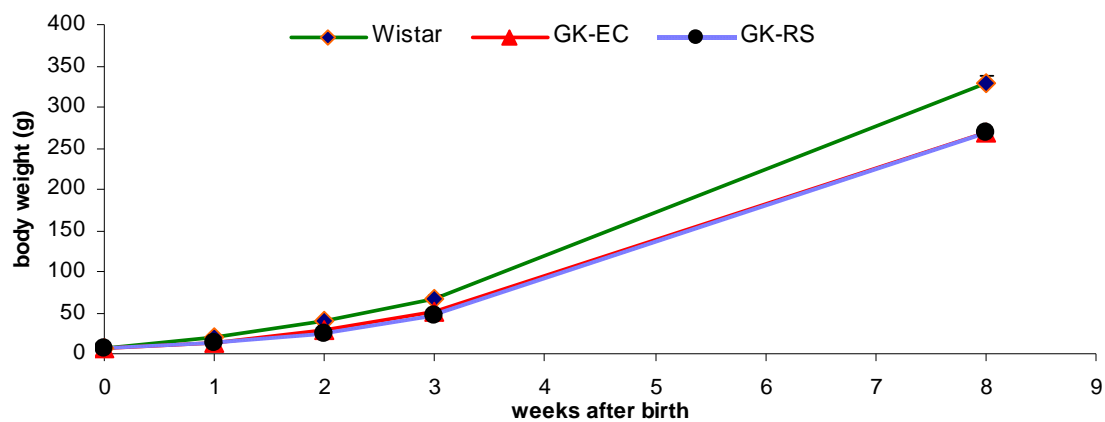


Figure 4.5 Growth curves of pups. No significant difference was detected between offspring of GK rats fed resistant starch and EC diet. Data are mean \pm SEM for group of 10 rats.

Table 4.1 Food intakes and disemboweled body weights of offspring

Group	Cumulative food intake (g)	Body weight (g)	Litter size
GK-EC	630.0 ± 10.3	268.7 ± 5.9	8.2 ± 1.4
GK-RS	596.1 ± 28.6	268.0 ± 7.3	9.11 ± 1.6

GK-EC: Pups born to GK rats fed control diet

GK-RS: Pups born to GK rats fed resistant starch diet

There were no significant difference in food intake, body weight and litter size between pups born to control and RS fed GK rats. Data are mean \pm SEM for group of 10 rats.

the two groups, so short chain fatty acids concentrations and microfloral population were not measured.

4.3.5 Food intake and Body Weight

There were no statistical differences in litter size between pups born to control and RS fed GK rats. It demonstrated no or minimal side effect on pregnancy with the consumption of resistant starch at the levels in their diet. No significant differences for body weight and food intake were found between offspring from control and RS fed GK rats (Table 4.1). No significant difference was detected in growth rate between offspring of GK rats fed resistant starch and EC diet (Figure 4.5).

4.4 Discussion

In this study, we investigated the effect of feeding dietary resistant starch to GK dams on glucose metabolism of their offspring. We demonstrate that offspring of GK rats fed resistant starch had lower fasting glucose levels and increased pancreatic insulin content compared with pups from dams fed the EC diet. In addition, we measured birth rate and growth of pups born to dams fed on both diets, and found there were no adverse effects observed on offspring when feeding resistant starch to dams.

As we predicted, there was no carry over fermentation that occurred in the hind gut of pups born to resistant starch fed GK dams which was evidenced by the similar cecal content weight and pH values obtained in both offspring. Therefore, the improvement of fasting glucose was not ascribed to postnatal elevation of short chain fatty acids, augmentation of GLP-1 and consequent enhanced insulin sensitivity. Moreover, we have shown that insulin sensitivity was not improved in pups of GK dams fed on resistant starch.

During the last few years, it has been suggested that the experience of hyperglycemia may contribute to an endocrine pancreas defect in the offspring (Simmons, 2006). Although the development of diabetes in GK rats results from both genetic and environmental determinants, Gauguier and coworkers (Gauguier et al, 1994) reported that offspring of GK females crossed with Wistar males had a more marked hyperglycemia than those of Wistar females crossed with GK males, suggesting a role of the intrauterine environment. However, not all studies agreed with this conclusion (Abdel-Halim et al., 1994). Gill-Randall and co-workers developed a rat embryo transfer technique to examine the weight of genetic factors and intrauterine hyperglycemia (Gill-Randall et al, 2004). They showed that Wistar embryos implanted into the uterus of GK mothers were more hyperglycemic in adulthood than those that were reared in Wistar mothers (Gill-Randall et al, 2004), this clearly illustrating the notion that intrauterine hyperglycemic environment is a risk factor for developing hyperglycemia in offspring at adulthood. Impaired gene expression and disturbed organogenesis attributed to increased oxidative stress (Loeken et al, 2006; Zhao et al, 2005) and reduced angiogenesis induced by hyperglycemia (Larger et al, 2004) are potential mechanisms associated with this anomaly. Tight glycemic control before conception and intensive glucose control maintained during pregnancy is suggested by ADA in order to reduce the prevalence of type 2 diabetes in offspring (American Diabetes Association, 2004).

In this study, we showed that feeding resistant starch to GK dams resulted in lower fasting glucose and enhanced pancreatic insulin content. These favorable effects may result from the improvement of maternal hyperglycemia which in turn changes the

intrauterine environment. Our findings provide evidence to apply resistant starch as an intervention in prevention and treatment of diabetes.

CHAPTER 5

CONCLUSIONS

The work in this dissertation focuses on the effects of dietary resistant starch on glycemic control in pregnant GK rats and the impact passed on to their offspring. We measured fat pad changes, fasting insulin and glucose concentration, insulin sensitivity, pancreatic insulin content and beta cell mass in the pregnant GK rats in the context of resistant starch feeding. We showed that feeding resistant starch significantly improved fasting glucose, increased insulin sensitivity and pancreatic beta cell mass in pregnant GK rats, a non obesity type 2 diabetes model. We further demonstrated that feeding resistant starch to pregnant GK rats decreased fasting glucose of their offspring without negative influences on growth and fetus survival rate.

We provide evidence to indicate that dietary resistant starch was able to improve pancreatic insulin content and beta cell mass in pregnant GK rats. The favorable effects of dietary resistant starch in pancreas islets might partly be through the elevation of GLP-1 which is stimulated by increased cecal short chain fatty acid resulted from promoting gut butyrate producing microbes with resistant starch. Further work is needed to for a conclusive determination for the role of increased GLP-1 in dietary resistant starch induced improvement on glycemic control in pregnant GK rats.

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VITA

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