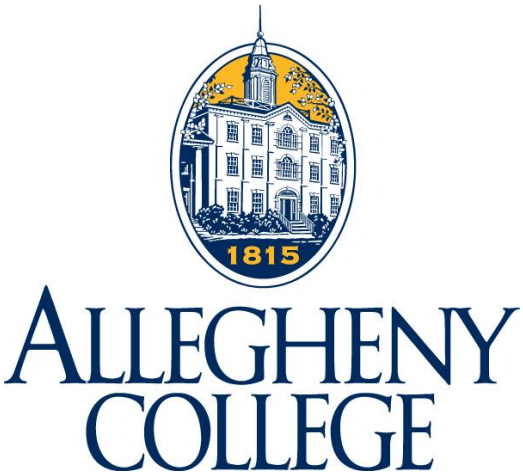
The effects of a high fat diet on blood glucose and glucose-6-phosphate dehydrogenase activity as markers for oxidative stress in mice



Claire DeLucia

Submitted to the Biology Department, Allegheny College
in partial fulfillment of the requirements for the degree of
BACHELOR OF SCIENCE

March 31, 2014

Pledge:

_____

Approved:

_____
Christine Donmoyer, Ph.D.

_____
Lee Coates, Ph.D.

Abstract

Oxidative stress via reactive oxygen species (ROS) and subsequent inflammation is a proposed mechanism for type I and II diabetes mellitus complications. The key characteristic of type II diabetes and hyperglycemia has been shown to decrease glucose-6-phosphate dehydrogenase (G6PDH) activity, the primary source of cellular NADPH. Through the recycling of glutathione disulfide (GSSG) into reduced glutathione (GSH), NADPH plays a critical role in antioxidant defense against ROS. The purpose of this study was to create a type II diabetes mouse model through a high fat diet and to observe the effects that diabetic hyperglycemia had on different steps of G6PDH-mediated antioxidant defense. Results indicate that a high fat diet is effective for inducing hyperglycemia in mice. Additionally, high blood glucose level significantly decreased G6PDH activity as well as NADPH/$NADP^+$ ratios in high fat diet fed mice. Male high fat diet mice also exhibited lower NADPH/$NADP^+$ ratios due to a decrease in G6PDH activity, although this trend was not seen both male and female mice. These results suggest that research of this nature needs to be conducted in more type II diabetes models considering 95% of those diagnosed with diabetes have type II.

ACKNOWLEDGEMENTS

My family: Words cannot describe how thankful I am to have you guys as my support system. Manny and Sam, you guys are two of the best brothers I could ever have. It has been amazing to watch you grow into kind, funny, responsible (sort of) young men. I know you will always have my back no matter what happens and that some quality sibling bonding can be as simple as watching The Office or Family Guy. Mom and Dad, I cannot even begin to thank you enough for all of the opportunities you have been able to present to me. From sending me to OC to helping me pay for my education here at Allegheny, you have always strived to provide the best for me and I appreciate every effort you have put forth thus far (the same goes for future ones, too). So I am dedicating my best work from my years at Allegheny to you.

You have all helped me through some of the best and worst years of my life and I hope you know how much I love you.

My friends: You guys know who you are and I am so happy that I have all of you in my life. Thank you for always being able to put a smile on my face and for teaching me that true friends don't run away when life gets difficult; they only hold on to you tighter until you can stand on your own again. I have enjoyed every moment we have spent together at Allegheny and the memories we have made here will last for years to come. I am going to miss all of you dearly but I know we all are going to be doing amazing things in years to come thanks to Allegheny and the support system we now have because of this place. Here's to the nights we spent laughing, crying and everything in between. Long live sports garden and walk-me-downs.

Dr. D: Thank you so much for the constant support from junior seminar to summer research and through the entire process of my comp. Your high expectations have only pushed me to become a better student and scientist. And just so you know, the day I turned in my comp was indeed the "best day ever."

My teammates: I never thought that I would ever be a track and field athlete in a million years. I eat, slept and played volleyball year-round since the age of 12. Thank you for being an amazing group of people and supporting me through the "consistently bad" years when I was just learning how to throw things, through my ACL tear and through an awesome 2013-2014 season. I am so happy I decided to join the throwing squad because I have laughed more and have been able to stay true to myself and participate in my favorite activity – athletics.

A special thanks goes to the **Allegheny College Biology department** for the funding of this senior project as well as the **Robertson Fund** for covering the additional costs. It is amazing to be a part of an institution where so many alumni donate to ensure the academic success of its students.

TABLE OF CONTENTS

Abstract……2

Acknowledgements……..3

Introduction…….….6

Methods……..10

ARC Approval……10

Animal Care……10

Feeding Protocol…….……11

Blood Glucose Measurement……..11

Animal Sacrifice and Tissue Collection…….…….…….……...……12

G6PDH Activity Assay…….…….……12

NADPH/$NADP^+$ Ratio…….……..12

Statistical Analysis…….13

Results……14

General Observations……...…….14

G6PDH Activity……14

NADPH/$NADP^+$ Ratio……..15

Figures……...……16

Discussion…….19

Type II Diabetic Mouse Model…….19

Effects of Hyperglycemia……..21

Fatty Liver Disease……22

Limitations…….22

Future Studies ……23

Conclusions……23

References……..24

Appendices…….28

Appendix A……29

Appendix B……30

Appendix C……31

Appendix D……35

Appendix E……38

Appendix F……40

Appendix G……42

Appendix H……44

Appendix I……..46

The effects of a high fat diet on blood glucose and glucose-6-phosphate dehydrogenase activity as markers for oxidative stress in mice

Approximately 8.3% (25.8 million people) of the United States population suffers from diabetes mellitus (diabetes.org). The common characteristic of diabetes is high blood glucose concentration, or hyperglycemia due to the body's failure to utilize and/or produce insulin. The β-cells of the pancreas are responsible for the production and secretion of insulin; if these cells are damaged or dysfunctional, the body has trouble keeping the blood glucose level within a normal range (Zhang et al. 2010). Insulin allows glucose from the blood stream to be taken up into muscle, fat and cardiac muscle cells to be utilized as fuel (Ye, 2013). When glucose is not taken into cells, it accumulates in the bloodstream and can cause detrimental complications such as neuropathy, retinopathy, and nephropathy.

The main types of diabetes mellitus that exist in humans are types I and II. Type I is classified as the inability for the body to produce insulin and only occurs in approximately 5% of diabetic patients. In the more prevalent type II, the body either does not produce enough insulin for homeostasis and/or cells cannot utilize the insulin being produced. This insulin resistance, in turn, does not allow the body to take in glucose as fuel for its cells (diabetes.org). Both types can lead to glucose accumulation in the blood stream and to further complications if not treated properly and promptly.

One enzyme that has been studied under diabetic conditions is the cytoplasmic enzyme glucose-6-phosphate dehydrogenase (G6PDH). G6PDH is best known as the rate-limiting enzyme in the pentose phosphate pathway (PPP), which produces nicotinamide adenine dinucleotide phosphate (NADPH) and ribose for nucleic acid synthesis (Mayes, 1993). NADPH is an important cofactor in many metabolic processes such as the production of cholesterol and

and lipids – a process that takes sugars and turns them into fats (Mayes, 1993). More importantly, NADPH assists in the glutathione reductase reaction, in which reduced glutathione (GSH) is produced through the recycling of glutathione disulfide (GSSG) (Birben et al., 2011; Maritim et al., 2002). GSH acts as an electron donor (antioxidant) to neutralize reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2) (Birben et al., 2011).

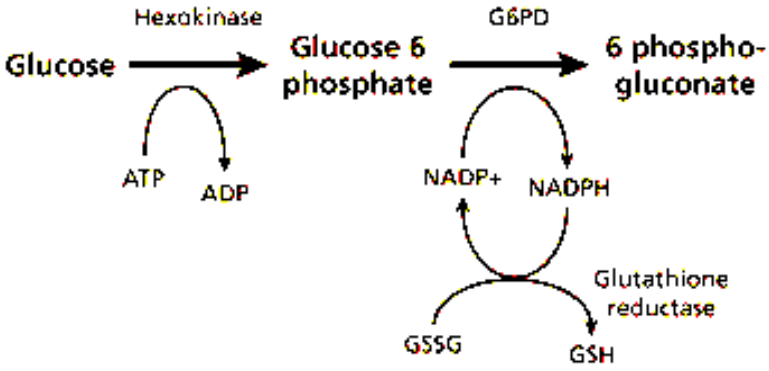


Figure 1. The mechanism of GSSG recycling via NADPH, a product of G6PDH (labeled "G6PD" in the figure. Picture taken from Frank 2005

Generated by mitochondria inside the cell, ROS are a normal part of cell homeostasis. But in high concentrations, ROS can severely affect structures and functions of lipids, proteins and DNA (Henrikson et al., 2011; Murphy 2008). The accumulation of ROS, along with a decrease of antioxidants, is termed oxidative stress (Birben et al., 2012). It has been proposed that oxidative stress is one of the factors that assist the progression of diabetic complications in areas such as the kidney and retina (Maritim et al., 2003). Additionally, the prevalence of oxidative stress in combination with other factors such as obesity, aging and fatty liver syndrome have been linked to the development of insulin resistance (Evans et al., 2003; Houstis et al., 2006), therefore increasing the risk for type II diabetes. Increases in cellular ROS have been linked to the inhibition of insulin-signaling pathways. The activation of factors such as tumor-necrosis factor-alpha (TNF-α) and nuclear factor-kappa beta (NF-κB) not only cause low-grade inflammation but are thought to mediate the degree to which insulin resistance occurs in an

individual (Ye, 2013; Houstis et al., 2006; Ogihara et al., 2004). Higher blood glucose levels are directly correlated with higher levels of insulin resistance.

Previous literature on rodents suggest that G6PDH activity decreases under hyperglycemic conditions (Carette et al., 2010; Prasath et al., 2011, Diaz-Flores et al., 2006). Although the mechanism behind this decrease in activity is not definitively known, one group has proposed a mechanism for this decreased activity. Zhang & Stanton (2000) found, in bovine aortic endothelial cells, that the increase in glucose concentration led to an increase in cyclic adenosine monophosphate (cAMP). This increase in cAMP level triggered increased activity of protein kinase A (PKA), which can phosphorylate G6PDH, therefore inhibiting the enzyme (Zhang & Stanton 2000).

Research has shown that when G6PDH is hindered by hyperglycemic conditions, oxidative damage will occur in cells that rely on GSH to fight oxidative stress. Since G6PDH activity determines the rate at which NADPH is produced, and NADPH is necessary to reduce GSSG to the antioxidant GSH, a lack of G6PDH activity can lead to higher levels of ROS and oxidative stress. Evidence of diabetic conditions (hyperglycemia) causing oxidative stress was reported in astroglia in the brain (Takahashi et al., 2011). That study found that an increase in glucose concentration in astroglia correlated with an increase in the amount of ROS. Additional evidence of the essential role of G6PDH in regards to oxidative stress is shown in another study conducted by Zhang et al. (2010) regarding the overexpression of G6PDH via adenoviral vector. This group concluded that oxidative stress due to hyperglycemic conditions was linked to β-cell apoptosis, or cell death. Overexpression of G6PDH decreased the frequency of apoptosis in β-cells, further supporting the hypothesis that G6PDH plays a crucial role in the pathway of managing ROS during oxidative stress (Zhang et al., 2010). Finally, decreased GSH

concentration has been associated with hyperglycemia in Wistar and Sprague-Dawley rats (Maritim et al., 2003). As the concentration of glucose in the bloodstream increases, the activity of G6PDH is hindered (reducing the amount of cellular NADPH), therefore decreasing the amount of GSH since NADPH is used to recycle GSH from GSSG.

Animal models of diabetes have been studied in regards to the disease and the biochemical effects it has on homeostasis. There are a variety of methods to either permanently or temporarily induce diabetic symptoms in mice and rats. Animal models of type I diabetes were used to observe the etiology of diabetes and could be achieved via pathogens, transgenic animals and pharmacological approaches (Van Belle et al., 2008). Although pathogenic and transgenic models have advantages, an injection of streptozotocin (STZ), a pharmacological approach, is a common procedure to induce type I diabetes in mice (Prasath et al., 2011; Tian et al., 2010; Graham et al., 2011; Diaz-Flores et al., 2006). This method creates a type I diabetic model, which represents only 5% of diabetic patients. I wanted to study the effects of diabetes on G6PDH, NADPH and GSH concentrations under more relevant conditions to type II diabetes. Therefore, a type II diabetic mouse model will be used in this project. While type II models can be surgically, pathogenically and genetically created (Srinivasan and Ramarao, 2007), type II hyperglycemia was induced nutritionally. A type II mouse model can be achieved with the aid of a high-fat diet (60% kcal from fat) over a set time period and can develop increased body weights, more insulin resistance as well as hyperglycemia (Winzell and Ahrén, 2004). The hyperglycemia seen in these animals is expected to decrease G6PDH activity and disrupt the ability to respond to oxidative stress.

This study was designed to measure G6PDH activity in diet-induced diabetes in the livers of C57BL/6J wild type mice. Using previous rat models as a guide (Diaz-Flores et al., 2006;

Prasath et al., 2011), G6PDH enzyme activity was measured in the liver under hyperglycemic conditions caused by a high-fat diet. By measuring G6PDH activity and the concentration of NADPH, conclusions can be drawn as to whether or not diabetes disrupts NADPH production via G6PDH and ultimately disrupts GSH antioxidant defense, a key player in reducing cellular ROS. It was hypothesized that the high fat diet group would have a decrease in G6PDH activity and NADPH concentration as well as higher blood glucose levels and body weights. If both G6PDH activity and NADPH concentration in the high-fat diet are lower than the control group, then it is appropriate to conclude that the diabetic group is experiencing more cellular oxidative stress, which could lead to complications.

Methods

ARC Approval

The Animal Research Committee (ARC) must approve all research projects involving animals at Allegheny College. The purpose of the committee is to ensure the ethical treatment of animals used for research. The feeding and euthaniasia protocols were submitted in writing to the committee, and this project was approved on October 9th, 2013.

Animal Care

Fourteen C57BL/6J mice from the colony of Allegheny College were used in this study. Males and females were housed separately upon weaning under a 12:12 light/dark cycle with free access to food. The mice were fed Laboratory Rodent Diet 5001 (5% of kcal from fat, LabDiet Inc., see Appendix A for composition) until 3 months of age and half of the mice

continued on this diet for 14 additional weeks. Laboratory Rodent Diet 5001 is the standard for all mice in Allegheny College.

Feeding Protocol

At approximately three months of age, the fourteen-week feeding program began. The mice were divided into two groups, high fat and regular diet. Half of the mice continued with the standard Laboratory Rodent Diet 5001 while the remaining half received a high fat diet (60% of kcal from fat diet, TD.06414, Appendix B) diet purchased from Harlan Laboratories. Males and females were assigned randomly to the two groups. The **regular diet** group consisted of four males and three females and the **high fat diet** group included three males and four females. Animals had free access to food and water until approximately four hours before sacrifice. Body weight was recorded (in grams) weekly to track weight gain. Food intake was also measured in grams.

Blood Glucose Measurement

After 14 weeks of feeding protocol, blood samples were taken from each mouse to measure glucose. This was to confirm hyperglycemia, a characteristic of type-II diabetes in the high-fat group. Animals were fasted 2 hours prior to collection. A drop of blood (approximately 10uL) was collected on a test strip from the tail vein via blood letting. The test strip was then inserted into a OneTouch® Ultra® 2 meter from LifeScan, Inc. for a glucose measurement. The expected blood glucose levels for the animals were 150 mg/dl for the regular diet mice and approximately 200 mg/dl for the high-fat mice (Keren et al., 2000).

Animal Sacrifice and Tissue Collection

Two days after blood glucose measurement, food was withheld four hours prior to sacrifice. Mice were euthanized in a chamber using CO_2 for two minutes. Whole livers were removed from the body cavity and placed in a 1.5 mL centrifuge tube. Tissue was then stored in a -70°C freezer until analysis. Two of the high fat diet male mice had fatty patches in the liver, and the organ was divided into two centrifuge tubes and stored.

G6PDH Activity Assay

After thawing, 20 mg samples from each liver were taken and placed on ice. These 20 mg samples were homogenized in 1mL ice-cold phosphate buffered saline (PBS) for approximately one minute until the tissue was completely dissolved. Samples were centrifuged at 15,000xg for 10 minutes at room temperature. During homogenization, some samples in the high fat group developed a layer of fat on top of the supernatant. Special care was taken to remove the fatty layer so the supernatant could be collected and used with the G6PDH assay kit purchased from Sigma Aldrich (See Appendix C for kit instructions) to measure G6PDH activity.

NADPH/$NADP^+$ Ratio

NADPH/$NADP^+$ was measured via the protocol as described by Zhang et al. (2000). First, a buffer solution of 1.0M Tris Base-HCl, 0.01M EDTA and 0.05% Triton X-100 (pH 8.0) was made. Then 20mg of liver tissue was homogenized in 1mL of buffer solution until dissolved. Samples were then centrifuged at 5,000xg for 7 minutes and the supernatants were collected. Specific reaction mixtures were made for the three spectrophotometer readings at 340 nm using the buffer solution, specific enzymes and their substrates. The first reading for each sample

consisted of 50μL of supernatant and 950μL of buffer solution. This measurement represented the total amount of NADPH and $NADP^+$ in the liver samples (A_1). A 50μL aliquot of the supernatant was then combined with 950μL of buffer solution that contained 5.0 IU G6PDH (Sigma Aldrich), 0.01M glucose-6-phosphate (Sigma Aldrich), and glucose-6-dehydrogenase to convert all of the $NADP^+$ in the sample to NADPH. This mixture was incubated at 37.5°C for five minutes and absorbance was taken (A_2). Similarly, another 50μL aliquot was combined with 950μL of buffer solution containing 5.0 IU glutathione reductase (Sigma Aldrich) and 0.005M glutathione (Sigma Aldrich) to convert the NADPH in the sample to $NADP^+$; absorbance was also measured (A_3). The following equations were used to determine the concentrations of NADPH, $NADP^+$ and the ratio of the two:

$A_1 - A_3$ = Total NADPH

$A_1 - A_2$ = Total $NADP^+$

Total NADPH/Total $NADP^+$ = Ratio

Statistical Analysis

The five main factors of interest in this study were final mouse weight, liver weight, blood glucose level, G6PDH activity and NADPH/$NADP^+$ ratio. Each of these factors were compared across sex and diet and tested for significance. All statistical analyses were performed using IBM SPSS Statistics (Version 19).

Two-tailed t-tests were done to determine if the high fat diet had significant effects on each of the five main factors. Equal variance was assumed for all cases. Additionally, Pearson Correlations were also performed to identify strong trends in the data that may not be statistically significant. SPSS t-test and Pearson Correlations outputs can all be found in Appendices E-I.

Results

General Observations

Mice weighed similar at beginning. After fourteen weeks of feeding with a regular or high fat diet, male mice had higher body weights than the female mice ($p=0.016$) (Table 1). The high fat diet significantly increased final body weight in the male mice ($p=0.008$) but not in female mice ($p=0.103$) (Figure 2). [how much did they gain?]

Average liver weights (Table 1) of animals on the high fat diet were not significantly different in female mice ($p=0.145$) but the male mice had heavier liver weights when on a high fat diet ($p=0.05$). Most notably, the livers of two of the high fat male mice were approximately twofold larger in size (area) than the control diet male group. Higher liver weights were also reported in male mice as compared to females ($p=0.034$).

Overall, diet had a hyperglycemic impact on blood glucose ($p=0.000$); high fat groups had higher blood glucose concentrations in both the male ($p=0.019$) and female ($p=0.007$) animals (Table 1). In addition to elevated body weight and liver weights, high fat animals of both sexes displayed greater visceral fat deposits than the control groups. Livers of both high fat fed groups were also lighter in color (pink) than control livers (dark red), an indication of fat accumulation (Figure 3).

G6PDH activity

The high fat diet and subsequent hyperglycemia did not significantly decrease overall G6PDH activity ($p=0.219$). Figure 4 shows the G6PDH activity of each group; decreased G6PDH activity is seen in the high fat diet fed animals. The high fat diet, associated with

hyperglycemia, is associated with lower G6PDH activity than the control group's activity in the male mice.

NADPH/$NADP^+$ ratio

Additionally, high fat diet-associated hyperglycemia did not have a significant impact on NADPH concentration (p=0.061). But Pearson correlation indicated that as blood glucose level increased, the NADPH/$NADP^+$ ratio decreased to a value less than 1, indicating a prevalence of cellular $NADP^+$ (p=0.019 Figure 5). Additionally, decreased G6PDH activity was not correlated with lower NADPH concentrations (p=0.377). Appendix O has the NADPH/$NADP^+$ ratios of high fat [and/] control mice. Ratios greater than 1 indicate more prevalence of cellular NADPH than $NADP^+$.

Table 1
Group characteristics with respect to sex and diet. Data reported as avg±SEM for each group. Body weight indicates the final weight of mouse 14 weeks after the start of the feeding protocol. Liver weights were weighed in 1.5mL centrifuge tubes. Blood glucose was measured in blood taken from the tail vein after 14 weeks of feeding protocol.
** Indicates significance ($p<0.05$) as compared to the control (regular diet) mice*

Group	Body Weight (g)	Liver Weight (g)	Blood glucose (mg/dL)
Control Male (n=4)	30.86±1.36	1.19±0.12	175.8±28.1
High Fat Male (n=3)	43.49±5.95*	2.93±1.20*	242.3±20.8*
Control Female (n=3)	24.09±0.59	0.99±0.22	151.0±14.7
High Fat Female (n=4)	29.27±4.37*	1.18±0.08	227.3±26.6*

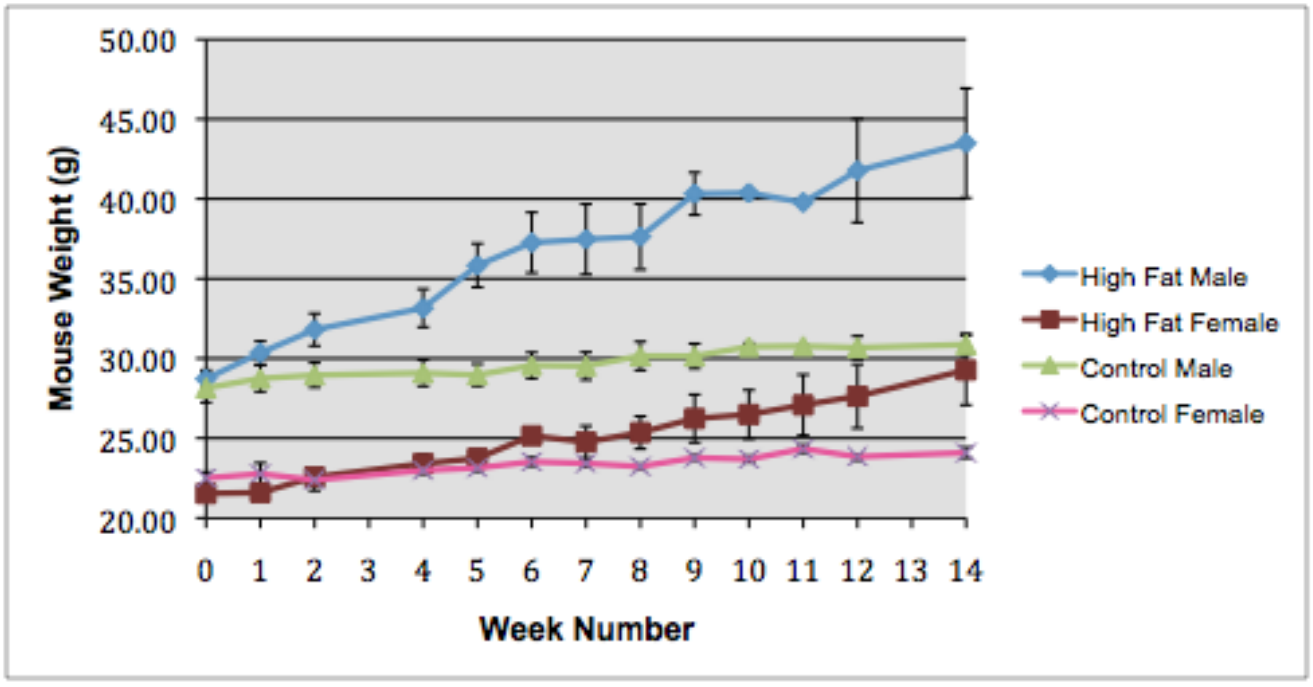


Figure 2. Average body weights of regular and high fat diet mice over a 14-week period. Error bars represent standard error.

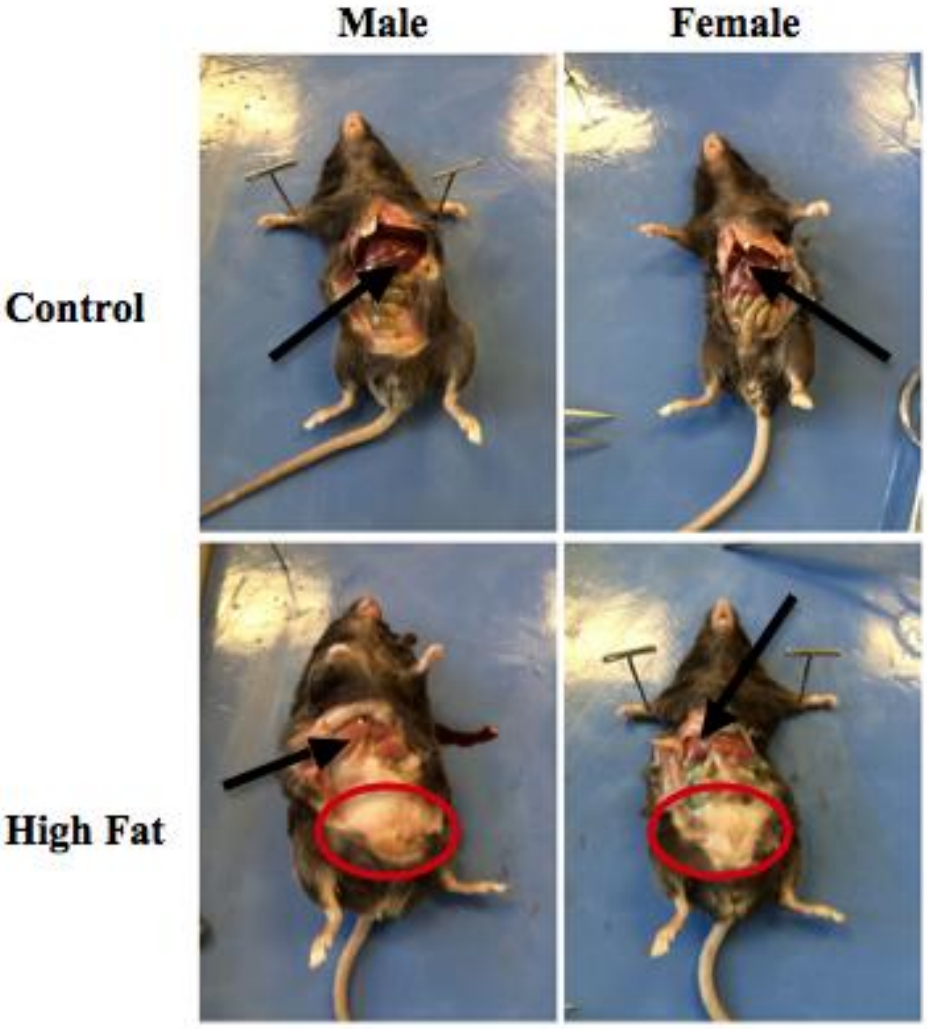


Figure 3. Ventral views of control and high fat mice. One male and one female of both diets are shown. Upon observation, the high fat group had more visceral fat than the control, especially in the lower abdominal region (circled in red). Black arrows identify livers. Note that the control livers are a deep red color while the high fat diet livers are a more pink color.

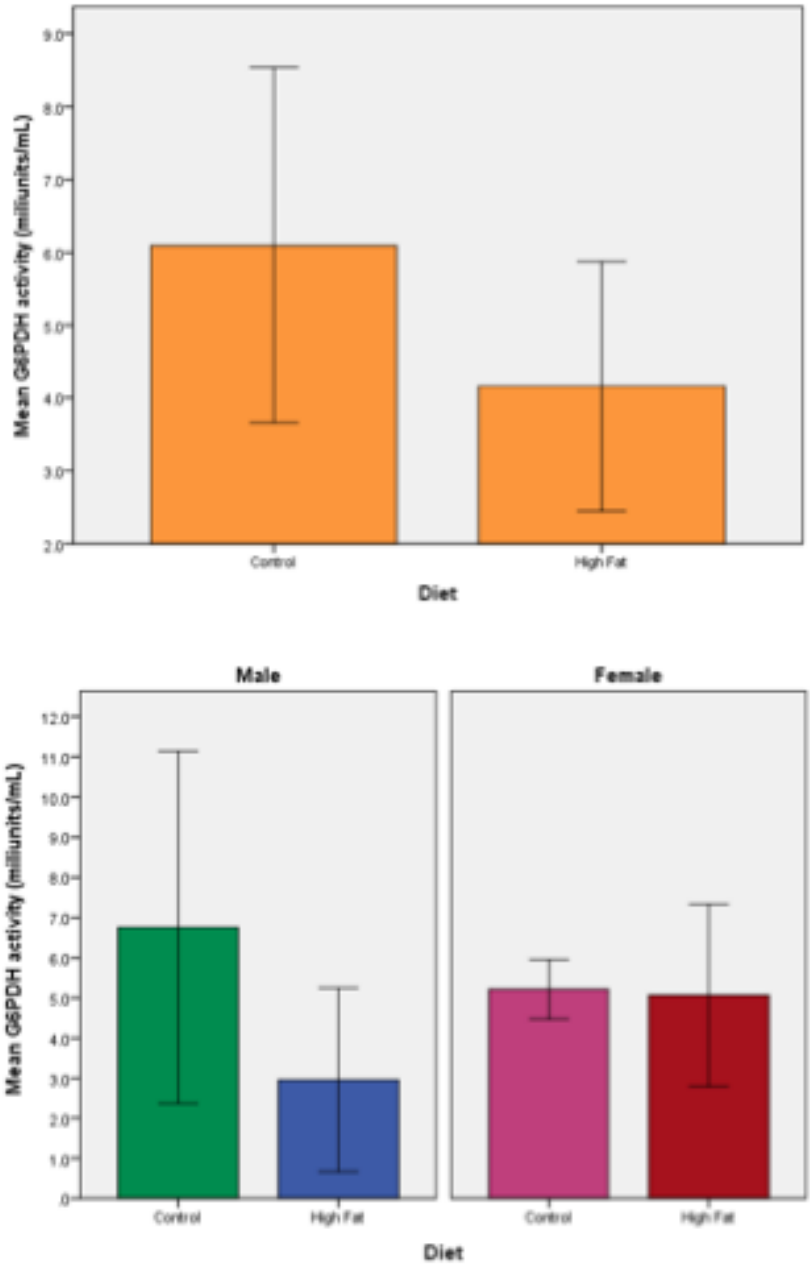


Figure 4. Average G6PDH activity in the liver of the high fat and control mice. Error bars represent standard error. (A) Average overall G6PDH activity of control and high fat diet mice. (B) Average overall G6PDH activity in each group

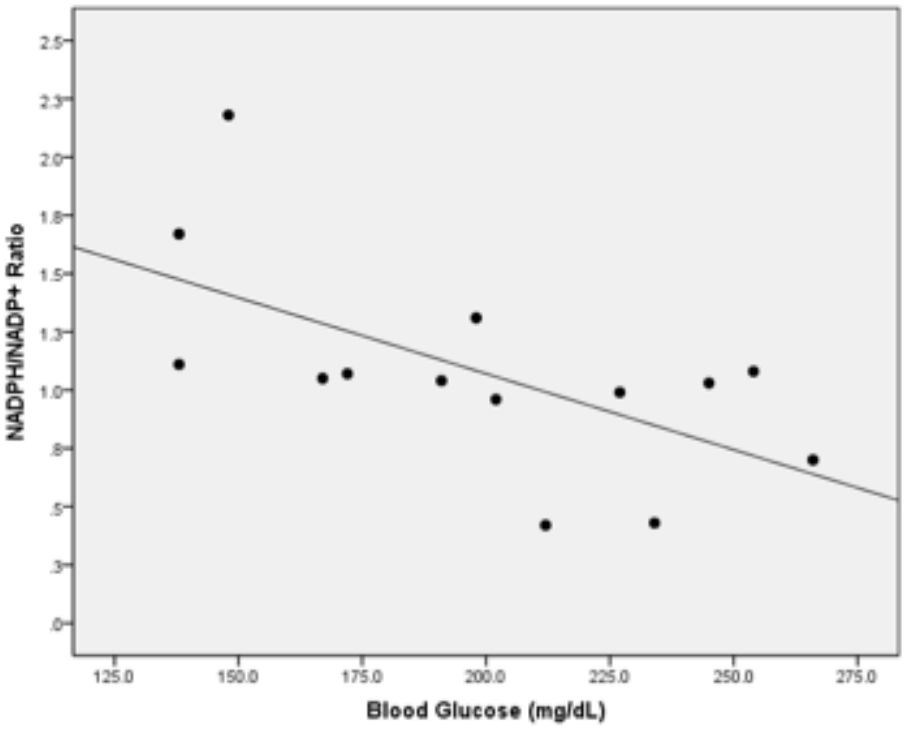


Figure 5. The negative linear relationship between blood glucose level and NADPH/NADP⁺ ratio. As blood glucose increases, the NADPH/NADP⁺ ratio decreases (p=0.016).

Discussion

Type II diabetic mouse model

The first goal of this study was to produce a type II diabetic model in mice by feeding a high fat diet over an extended period of time. In contrast, Previous literature studying antioxidant defense under hyperglycemic conditions suggest chemically inducing diabetic symptoms. Different mutagens such as alloxan (Mohan and Das, 1998) or STZ (Díaz-Flores et al., 2006; Rauscher et al., 2001; Sanders et al., 2000) can destroy β-cells in the pancreas, completely inhibiting the production of insulin. Without the regulatory action of insulin in the bloodstream, plasma glucose concentration can increase, putting an individual at risk for diabetic

complications. However, complete destruction of β-cells eliminates all insulin production, a characteristic of type I diabetes, which is experienced by approximately 5% of individuals suffering from diabetes mellitus (diabetes.org). Therefore, models that use chemical mutagens are more representative of type I rather than type II diabetes. While genetic, pathological and surgically-induced models of the prevalent type II diabetes exist (Srinivasan and Ramarao, 2007), these models do not represent the common risk factor associated with type II diabetes – increased body weight from food intake. Winzell & Ahren (2004) successfully created a type II diabetes mouse model by feeding young mice a high fat diet for 14 weeks. This model is a better representation of the human type II diabetic population since the risk of developing type II is increased with higher body mass index (BMI), an assessment of excess body fat (Van Greevenbroek et al., 2013).

Early results of this study indicated that the attempt to create a type II diabetes mouse model was successful. Blood glucose levels of both male and female high fat groups were significantly higher than the regular diet control (Table 1). A trend for higher average body weights were observed in the high fat diet group than the regular diet group (Figure 2). Although the differences in body weight between diets did not reach significance overall ($p=0.062$), the male high fat diet mice were significantly heavier than regular diet mice ($p=0.008$).

Additionally, diet had an impact on blood glucose level in male ($p=0.019$) and female ($p=0.007$) groups. Mice that were fed the high fat diet (60% kcal fat) had significantly higher blood glucose levels than the mice that were fed the regular diet (5% kcal from fat) ($p=0.000$, Table 1). Hyperglycemia is the cornerstone characteristic of diabetes mellitus (types I and II) as well as the main symptom observed in this study via the high fat diet. Hyperglycemia in the high fat diet was necessary for the success of this study.

Effects of hyperglycemia

The second goal of this project was to observe the effects of hyperglycemia on G6PDH activity and NADPH concentration in the liver. G6PDH, the rate-limiting enzyme of the PPP, has been identified as the primary generator of NADPH in cells (Xu et al., 2005). This means that if G6PDH efficiency is attenuated, the production of the reducing agent NADPH will also decrease. Because NADPH is used to reduce GSSG to the antioxidant GSH (Mayes, 1993), lower amounts of NADPH can lead to accumulation of ROS. Previous studies in rats (Díaz-Flores et al., 2006; Xu et al., 2005), mice (Zhang et al., 2010) and cell cultures (Asahina et al., 1995; Takahashi et al., 2011; Zhang & Stanton 2000) have shown that G6PDH activity is inhibited under hyperglycemic conditions, presumably because of the decreased activity of the enzyme.

It was hypothesized that the resulting hyperglycemia from the high fat diet would decrease G6PDH activity in the liver and consequently the cellular NADPH concentration. Results of this study indicated that blood glucose did not significantly affect overall G6PDH activity. A strong trend was found between higher blood glucose levels and lower G6PDH activity, however, possibly indicating that higher levels of glucose inhibit G6PDH. However, G6PDH activity did not significantly reduce NADPH concentration ($p=0.911$), an unexpected result. These data suggest that NADPH may be produced by another source that is also affected by hyperglycemic conditions, since blood glucose still had a significant effect on NADPH concentration while G6PDH activity did not.

Fatty liver disease

Another noteworthy finding of this study was the increased size and body weight of two of the three male mice in the high fat diet group. High fat diet male mouse 1 (HFM1) and high fat diet male mouse 2 (HFM2) both had light pink livers with fatty deposits. These fatty deposits are classified as ectopic fat and are a risk factor for metabolic diseases (such as type II diabetes) and can lead to non-alcoholic fatty liver disease (NAFLD) (Van Greevenbroek et al., 2013). NAFLD is known as a diabetic precursor and can act as the "first hit" in the development of type II diabetes. After the accumulation of fat in the liver, oxidative stress and subsequent inflammation of hepatocytes can activate TNF-α, triggering insulin resistance and development of type II diabetes and hyperglycemia (Hsieh and Hsieh, 2011).

Limitations

Although the high fat diet significantly increased blood glucose levels and was strongly correlated to higher body weights, assessing only these two factors does not provide a true type II diabetic mouse model. A more powerful type II diabetic mouse model would have been created if insulin had been measured and higher levels were found in the high fat diet group.

Additionally, time constraints limited this project. Mice were fed the high fat diet for 14 weeks, a relatively short period of time. If feeding was extended 6 weeks was in this study, more significant effects of the high fat diet may be observed in blood glucose level, G6PDH activity and $NADPH/NADP^+$ ratio shifts. By extending the feeding time frame, the high fat group could gain more weight, hopefully accompanied by significant differences in blood glucose levels and G6PDH activity since the hyperglycemic conditions would have proliferated for a longer period

of time than what was available for this study. The high fat diet group at 14 weeks may have been in the early stages of type II diabetes and therefore not yet experiencing the negative implications (oxidative stress) of high blood glucose levels.

This study used male and female mice, but results of this study indicated that male mice were more susceptible to the negative effects of the high fat diet than were female mice. This could be because estrogen plays a role in blood glucose homeostasis (Ropero et al., 2008), interfering with the rise in blood glucose induced by the high fat diet. Estrogen receptor alpha (ERα) is associated with glucose metabolism in the skeletal muscle, liver and brain. It is also involved with mediating liver insulin sensitivity (Ropero et al., 2008). Since females have higher levels of estrogen, this ERα receptor could function properly and modulate blood glucose levels back down to more normal levels in the female mice. If this study were repeated, only male mice would be used so the effects of estrogen on blood glucose level would not be a confounding factor.

G6PDH activity and NADPH concentration were the only two components of the GSH antioxidant defense pathway that were analyzed. Although G6PDH activity and NADPH concentration were decreased in high fat diet group, the antioxidant GSH was not measured, so a true estimate of antioxidant defense was not performed. Since GSH is the component of the pathway that combats ROS, measuring GSH concentration would allow for a better estimate of oxidative stress in the liver.

Future Studies

Since GSH was not measured, a future study would be to measure GSH levels in this type II diabetic mouse model. Because GSH more directly interacts with ROS, an increase of GSH in

tissues could indicate an elevated level of oxidative stress. Another direction would be to examine other markers of oxidative stress. Levels of superoxide (O_2^-) or hydrogen peroxide (H_2O_2) in a diabetic mouse model would be compared to a control. If the concentration of cellular ROS O_2^- and H_2O_2 increase in the hyperglycemic conditions, then diabetic animals would be experiencing higher levels of oxidative stress.

Conclusions

Feeding a high fat diet (60% kcal from fat) for 14 weeks was sufficient in creating a type II diabetic mouse model with the key symptom of the disorder – hyperglycemia. Under hyperglycemic conditions, G6PDH activity and NADPH concentration both decreased, the decrease of G6PDH activity, however may not have been the underlying cause of the NADPH concentration decrease. The decreased enzyme activity and NADPH concentration do, however, indicate that the high fat diet mice may have more exposure to oxidative stress. Most notably, male mice with the high fat diet accumulated fatty deposits in the liver, which could indicate precursors to non-alcoholic fatty liver disease and an advanced progression of type II diabetes.

References

Asahina T, Kashiwagi A, Nishio Y, et al. 1995. Impaired activation of glucose oxidation and NADPH supply in human endothelial cells exposed to H2O2 in high-glucose medium. *Diabetes* 44:520–6.

Birben E, Sahiner U, Sackesen C, Erzurum S, Kalayci O. 2011. Oxidative stress and antioxidant defense. *The World Allergy Organization journal* 5:9–19.

Carette C, Dubois-Laforgue D, Gautier J-F, Timsit J. 2011. Diabetes mellitus and glucose-6-phosphate dehydrogenase deficiency: from one crisis to another. *Diabetes & metabolism* 37:79–82.

Díaz-Flores M, Ibáñez-Hernández M, Galván R, et al. 2006. Glucose-6-phosphate dehydrogenase activity and NADPH/NADP+ ratio in liver and pancreas are dependent on the severity of hyperglycemia in rat. *Life sciences* 78:2601–7.

Evans J, Goldfine I, Maddux B, Grodsky G. 2002. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? *Diabetes* 52:1–8.

Frank J. 2005. Diagnosis and management of G6PD deficiency. *American family physician* 72:1277–82.

Graham M, Janecek J, Kittredge J, Hering B, Schuurman H-J. 2011. The streptozotocin-induced diabetic nude mouse model: differences between animals from different sources. *Comparative medicine 61*:356–60.

Henriksen E, Diamond-Stanic M, Marchionne E. 2011. Oxidative stress and the etiology of insulin resistance and type 2 diabetes. *Free radical biology & medicine* 51:993–9.

Houstis N, Rosen E, Lander E. 2006. Reactive oxygen species have a causal role in multiple forms of insulin resistance. *Nature* 440:944–8.

Hsieh P-S, Hsieh Y-J. 2011. Impact of liver diseases on the development of type 2 diabetes mellitus. *World journal of gastroenterology : WJG* 17:5240–5.

Keren P, George J, Shaish A, et al. 2000. Effect of hyperglycemia and hyperlipidemia on atherosclerosis in LDL receptor-deficient mice: establishment of a combined model and association with heat shock protein 65 immunity. *Diabetes* 49:1064–9.

Keren P, George J, Shaish A, et al. 2000. Effect of hyperglycemia and hyperlipidemia on atherosclerosis in LDL receptor-deficient mice: establishment of a combined model and association with heat shock protein 65 immunity. *Diabetes* 49:1064–9.

Maritim A, Sanders R, Watkins J. 2002. Diabetes, oxidative stress, and antioxidants: a review. *Journal of biochemical and molecular toxicology* 17:24–38.

Mayes, P. A. (1993). The pentose phosphate pathway & other pathways of hexose metabolism. In R. K. Murray, D. K. Granner, P. A. Mayes & V. W. Rodwell (Eds.), *Harper's biochemistry* (23rd ed., pp. 201-211). Norwalk, CT: Appleton & Lange.

Mohan I, Das U. 1998. Effect of L-arginine-nitric oxide system on chemical-induced diabetes mellitus. *Free radical biology & medicine* 25:757–65.

Murphy M. 2008. How mitochondria produce reactive oxygen species. *The Biochemical journal* 417:1–13.

Ogihara T, Asano T, Katagiri H, et al. 2004. Oxidative stress induces insulin resistance by activating the nuclear factor-kappa B pathway and disrupting normal subcellular distribution of phosphatidylinositol 3-kinase. *Diabetologia* 47:794–805.

Prasath G, Subramanian S. 2011. Modulatory effects of fisetin, a bioflavonoid, on hyperglycemia by attenuating the key enzymes of carbohydrate metabolism in hepatic and renal tissues in streptozotocin-induced diabetic rats. *European journal of pharmacology* 668:492–6.

Rauscher F, Sanders R, Watkins J. 2000. Effects of isoeugenol on oxidative stress pathways in normal and streptozotocin-induced diabetic rats. *Journal of biochemical and molecular toxicology* 15:159–64.

Ropero A, Alonso-Magdalena P, Quesada I, Nadal A. 2008. The role of estrogen receptors in the control of energy and glucose homeostasis. *Steroids* 73:874–9.

Sanders R, Rauscher F, Watkins J. 2000. Effects of quercetin on antioxidant defense in streptozotocin-induced diabetic rats. *Journal of biochemical and molecular toxicology* 15:143–9.

Srinivasan K, Ramarao P. 2007. Animal models in type 2 diabetes research: an overview. *The Indian journal of medical research* 125:451–72.

Takahashi S, Izawa Y, Suzuki N. 2011. Astroglial pentose phosphate pathway rates in response to high-glucose environments. *ASN neuro* 4.

Tian H, Wei L, Xu Z, Zhao R, Jin D, Gao J. 2010. Correlations between blood glucose level and diabetes signs in streptozotocin induced diabetic mice. *Global J Pharmacol* 4:111–116.

Van Belle T, Taylor P, von Herrath M. 2008. Mouse Models for Type 1 Diabetes. Drug discovery today. *Disease models* 6:41–45.

Van Greevenbroek M, Schalkwijk C, Stehouwer C. 2013. Obesity-associated low-grade inflammation in type 2 diabetes mellitus: causes and consequences. *The Netherlands journal of medicine* 71:174–87.

Winzell M, Ahrén B. 2004. The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes* 53 Suppl 3:S215–9.

Xu Y, Osborne B, Stanton R. 2005. Diabetes causes inhibition of glucose-6-phosphate dehydrogenase via activation of PKA, which contributes to oxidative stress in rat kidney cortex. American journal of physiology. *Renal physiology* 289:F1040–7.

Ye J. 2013. Mechanisms of insulin resistance in obesity. *Frontiers of medicine* 7:14–24.

Zhang Z, Apse K, Pang J, Stanton R. 2000. High glucose inhibits glucose-6-phosphate dehydrogenase via cAMP in aortic endothelial cells. *The Journal of biological chemistry* 275:40042–7.

Zhang Z, Liew C, Handy D, et al. 2010. High glucose inhibits glucose-6-phosphate dehydrogenase, leading to increased oxidative stress and beta-cell apoptosis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 24:1497–505.

Zhang Z, Yu J, Stanton R. 2000. A method for determination of pyridine nucleotides using a single extract. *Analytical biochemistry* 285:163–7.

Appendices

A: Regular mouse diet; Laboratory Rodent Diet 5001 – LabDiet, Inc.

B: High fat (60% kcal from fat) mouse diet; TD.06414 - Harlan Laboratories

C: G6PDH assay protocol – Sigma Aldrich

D: Raw data

E: SPSS output – Independent samples t-test; Sex and final weight, blood glucose, G6PDH activity, NADPH/$NADP^+$ ratio and liver weight

F: SPSS output – Independent samples t-test; diet and final weight, blood glucose, G6PDH activity, NADPH/$NADP^+$ ratio and liver weight

G: SPSS output – Independent samples t-test; Male diet and final weight, blood glucose, G6PDH activity, NADPH/$NADP^+$ ratio and liver weight

H: SPSS output – Independent samples t-test; Female diet and final weight, blood glucose, G6PDH activity, NADPH/$NADP^+$ ratio and liver weight

I: SPSS output – Independent samples t-test; Pearson Correlations

APPENDIX A

Laboratory Rodent Diet 5001*

DESCRIPTION

Laboratory Rodent Diet is recommended for rats, mice, hamsters and gerbils. This diet is a complete life cycle diet formulated using managed formulation, delivering Constant Nutrition®. This is paired with the selection of highest quality ingredients to assure minimal inherent biological variation in long-term studies. It is formulated for life-cycle nutrition; however, it is not designed for maximizing production in mouse breeding colonies. This product has been the standard of biomedical research for over 70 years.

Features and Benefits

- Managed Formulation delivers Constant Nutrition®
- High quality animal protein added to create a superior balance of amino acids for optimum performance
- Formulated for multiple species for single product inventory
- The rodent diet standard for biomedical research

Product Forms Available

- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")
- Meal (ground pellets)

Other Versions Available

- 5L0D PicoLab Laboratory Rodent Diet (Minimum order required)

GUARANTEED ANALYSIS

Crude protein not less than	23.0%
Crude fat not less than	4.5%
Crude fiber not more than	6.0%
Ash not more than	8.0%

INGREDIENTS

Ground corn, dehulled soybean meal, dried beet pulp, fish meal, ground oats, brewers dried yeast, cane molasses, dehydrated alfalfa meal, dried whey, wheat germ, porcine animal fat preserved with BHA, porcine meat meal, wheat middlings, salt, calcium carbonate, DL-methionine, choline chloride, cholecalciferol, vitamin A acetate, folic acid, menadione dimethylpyrimidinol bisulfite (source of vitamin K), pyridoxine hydrochloride, biotin, thiamin mononitrate, nicotinic acid, calcium pantothenate, dl-alpha tocopheryl acetate, vitamin B_{12} supplement, riboflavin, ferrous sulfate, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate, sodium selenite.

FEEDING DIRECTIONS

Feed ad libitum to rodents. Plenty of fresh, clean water should be available to the animals at all times.

Rats- All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat up to 30 grams per day. Smaller strains will eat up to 15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.

Mice-Adult mice will eat up to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

Hamsters-Adults will eat up to 14 grams per day.

For information regarding shelf life please visit www.labdiet.com.

06/20/13

CHEMICAL COMPOSITION[1]

Nutrients[2]	
Protein, %	**23.9**
Arginine, %	1.41
Cystine, %	0.31
Glycine, %	1.21
Histidine, %	0.57
Isoleucine, %	1.14
Leucine, %	1.83
Lysine, %	1.41
Methionine, %	0.67
Phenylalanine, %	1.04
Tyrosine, %	0.71
Threonine, %	0.91
Tryptophan, %	0.29
Valine, %	1.17
Serine, %	1.19
Aspartic Acid, %	2.81
Glutamic Acid, %	4.37
Alanine, %	1.43
Proline, %	1.49
Taurine, %	0.02
Fat (ether extract), %	**5.0**
Fat (acid hydrolysis), %	**5.7**
Cholesterol, ppm	200
Linoleic Acid, %	1.22
Linolenic Acid, %	0.10
Arachidonic Acid, %	<0.01
Omega-3 Fatty Acids, %	0.19
Total Saturated Fatty Acids, %	1.56
Total Monounsaturated Fatty Acids, %	1.60
Fiber (Crude), %	**5.1**
Neutral Detergent Fiber[3], %	15.6
Acid Detergent Fiber[4], %	6.7
Nitrogen-Free Extract (by difference), %	**48.7**
Starch, %	31.9
Glucose, %	0.22
Fructose, %	0.30
Sucrose, %	3.70
Lactose, %	2.01
Total Digestible Nutrients, %	**76.0**
Gross Energy, kcal/gm	**4.07**
Physiological Fuel Value[5], kcal/gm	**3.36**
Metabolizable Energy, kcal/gm	**3.02**

Minerals	
Ash, %	**7.0**
Calcium, %	0.95
Phosphorus, %	0.66
Phosphorus (non-phytate), %	0.39
Potassium, %	1.18
Magnesium, %	0.21
Sulfur, %	0.36
Sodium, %	0.40
Chloride, %	0.67
Fluorine, ppm	16
Iron, ppm	270
Zinc, ppm	79
Manganese, ppm	70
Copper, ppm	13
Cobalt, ppm	0.90
Iodine, ppm	1.0
Chromium, ppm	1.2
Selenium, ppm	0.30

Vitamins	
Carotene, ppm	2.3
Vitamin K (as menadione), ppm	1.3
Thiamin Hydrochloride, ppm	16
Riboflavin, ppm	4.5
Niacin, ppm	120
Pantothenic Acid, ppm	24
Choline Chloride, ppm	2250
Folic Acid, ppm	7.1
Pyridoxine, ppm	6.0
Biotin, ppm	0.30
B_{12}, mcg/kg	50
Vitamin A, IU/gm	15
Vitamin D_3 (added), IU/gm	4.5
Vitamin E, IU/kg	42
Ascorbic Acid, mg/gm	—

Calories provided by:	
Protein, %	28.507
Fat (ether extract), %	13.496
Carbohydrates, %	57.996

*Product Code

1. Formulation based on calculated values from the latest ingredient analysis information. Since nutrient composition of natural ingredients varies and some nutrient loss will occur due to manufacturing processes, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

LabDiet
www.labdiet.com

APPENDIX B

Teklad Custom Research Diet Data Sheet

TD.06414 Adjusted Calories Diet (60/Fat)

Formula	g/Kg
Casein	265.0
L-Cystine	4.0
Maltodextrin	160.0
Sucrose	90.0
Lard	310.0
Soybean Oil	30.0
Cellulose	65.5
Mineral Mix, AIN-93G-MX (94046)	48.0
Calcium Phosphate, dibasic	3.4
Vitamin Mix, AIN-93-VX (94047)	21.0
Choline Bitartrate	3.0
Blue Food Color	0.1

Footnote

Approx. 60% of total calories come from fat. Designed with similarities to Research Diets, Inc. formula D12492. For the series TD 06414-TD 06416. Approximate fatty acid profile (% of total fat): 37% saturated, 47% monounsaturated, 16% polyunsaturated.

Selected Nutrient Information[1]

	% by weight	% kcal from
Protein	23.5	18.4
Carbohydrate	27.3	21.3
Fat	34.3	60.3

Kcal/g 5.1

[1] Values are calculated from ingredient analysis or manufacturer data

Teklad Diets are designed & manufactured for research purposes only.

Speak With A Nutritionist

- **(800) 483-5523**
- **askanutritionist@harlan.com**

harlan™

Harlan Laboratories · PO Box 44220 · Madison, WI 53744-4220
www.harlan.com

Key Features

- **Purified Diet**
- **Diet Induced Obesity**
- **High Fat**

Key Planning Information

- *Products are made fresh to order*
- Store product at 4°C or lower
- Use within 6 months (applicable to most diets)
- Box labeled with product name, manufacturing date, and lot number
- Replace diet at minimum once per week
  More frequent replacement may be advised
- Lead time:
  - 2 weeks non-irradiated
  - 4 weeks irradiated

Product Specific Information

- 1/2" Pellet or Powder (free flowing)
- Minimum order 3 Kg
- Irradiation available upon request

Options (Fees Will Apply)

- Rush order (pending availability)
- Irradiation (see Product Specific Information)
- Vacuum packaging (1 and 2 Kg)

International Inquiry

-Outside U.S.A. or Canada-

- **askanutritionist@harlan.com**

Place Your Order (U.S.A. & Canada)

· Place Order · Obtain Pricing ·
· Check Order Status ·

- **(800) 483-5523**
- **(608) 277-2066** *facsimile*
- **tekladinfo@harlan.com**

Helping you do research better

Harlan, Harlan Laboratories, Helping you do research better, and the Harlan logo are trademarks and trade names of Harlan Laboratories, Inc.
©2009 Harlan Laboratories, Inc.

APPENDIX C

SIGMA-ALDRICH®

sigma-aldrich.com

3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Glucose-6-Phosphate Dehydrogenase Assay Kit

Catalog Number **MAK015**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description
Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the conversion of glucose-6-phosphate to 6-phosphoglucono-δ-lactone, the first and rate-limiting step of the pentose phosphate pathway (PPP). The PPP pathway is critical for maintaining the cofactor nicotinamide adenine dinucleotide phosphate (NADPH) and for the production of pentose sugars. The NADPH produced is critical for redox regulation via the regeneration of GSH and for providing reducing equivalents for fatty acid biosynthesis. Deficiencies in G6PDH predisposes individuals to non-immune hemolytic anemia.

The Glucose-6-Phosphate Dehydrogenase Assay Kit is a simple, sensitive and rapid assay detects the activity of G6PDH in a variety of samples. In this kit, glucose-6-phosphate is oxidized to generate a product, which is specifically detected by colorimetric (450 nm) assay. The G6PDH Assay Kit can detect as low as 0.04 milliunit of G6PDH per well.

Components
The kit is sufficient for 100 assays in 96 well plates.

G6PDH Assay Buffer Catalog Number MAK015A	25 mL
G6PDH Substrate Catalog Number MAK015B	1 vl
G6PDH Developer Catalog Number MAK015C	1 vl
G6PDH Positive Control Catalog Number MAK015D	1 vl
NADH Standard, 0.5 µmole Catalog Number MAK015E	1 vl

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

G6PDH Assay Buffer – Allow buffer to come to room temperature before use.

G6PDH Substrate Mix – Reconstitute in 220 µL of G6PDH Assay Buffer. Mix well by pipetting. Once reconstituted, the Substrate Mix is stable for two months at 4 °C.

G6PDH Developer – Reconstitute in 220 µL of water. Mix well by pipetting, and then aliquot and store at –20 °C. Use within 2 months of reconstitution.

G6PDH Positive Control – Reconstitute in 100 µL of G6PDH Assay Buffer. Mix well by pipetting and keep cold while in use. Aliquot and store at –20 °C. Use within 2 months of reconstitution.

NADH Standard – Reconstitute in 400 µL of water to generate a 1.25 mM standard solution. Mix well by pipetting and keep cold while in use. Aliquot and store at –20 °C. Use within 2 months of reconstitution.

Storage/Stability
The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

2

Procedure
All samples and standards should be run in duplicate.

NADH Standards for Colorimetric Detection
Add 0, 2, 4, 6, 8, and 10 µL of the 1.25 mM NADH standard solution in duplicate into a 96 well plate, generating 0 (blank), 2.5, 5, 7.5, 10, and 12.5 nmole/well standards. Add Assay Buffer to a final volume of 50 µL.

Sample Preparation
Tissue (10–20 mg), cells (1×10^6), or erythrocyte samples (10–100 mg) should be rapidly homogenized in equivalent volumes of ice cold PBS or other buffer (pH 6.5–8). Centrifuge at 15,000 × *g* for 10 minutes to remove insoluble materials. Add 1–50 µL samples into duplicate wells of a 96-well plate. Bring samples to a final volume of 50 µL with G6PDH Assay Buffer.

For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range.

Dilute 10 µL of the Positive Control with 990 µL of Assay Buffer. Add 1–10 µL of the diluted Positive Control into duplicate wells of 96 well plate. Bring to final volume of 50 µL with G6PDH Assay Buffer. This should be a suitable dilution to get 0.1–1.0 A_{450} in 30 minutes of incubation.

Assay Reaction
1. Set up the Master Reaction Mix according to the scheme in Table 1. 50 µL of the Master Reaction Mix is required for each reaction (well).

Table 1.
Master Reaction Mix

Reagent	Master Reaction Mix
G6PDH Assay Buffer	46 µL
G6PDH Substrate Mix	2 µL
G6PDH Developer Mix	2 µL

2. Add 50 µL of the Master Reaction Mix to each of the standard, positive control, and sample wells. Protect the plate from light. Mix well using a horizontal shaker or by pipetting.

3. After 2–3 minutes, take the initial measurement ($T_{initial}$). Measure the absorbance at 450 nm at the initial time $(A_{450})_{initial}$.
Note: It is essential $(A_{450})_{initial}$ is in the linear range of the standard curve.

4. Incubate the plate at 37 °C taking measurements (A_{450}) every 5 minutes. Protect the plate from light during the incubation.

5. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (12.5 nmole/well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve.

6. The final measurement $[(A_{450})_{final}]$ for calculating the enzyme activity would be penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve, see step 5. The time of the penultimate reading is T_{final}.
Note: It is essential the final measurement falls within the linear range of the standard curve.

Results

Calculations

Correct for the background by subtracting the final measurement [$(A_{450})_{final}$] obtained for the 0 (blank) NADH standard from the final measurement [$(A_{450})_{final}$] of the standards. Background values can be significant and must be subtracted from all readings. Plot the NADH standard curve.

Note: A new standard curve must be set up each time the assay is run.

Calculate the change in measurement from $T_{initial}$ to T_{final} for the samples.

$$\Delta A_{450} = (A_{450})_{final} - (A_{450})_{initial}$$

Compare the ΔA_{450} of each sample to the standard curve to determine the amount of NADH generated by the kinase assay between $T_{initial}$ and T_{final} (B).

The G6PDH activity of a sample may be determined by the following equation:

$$\text{G6PDH Activity} = \frac{B \times \text{Sample Dilution Factor}}{(\text{Reaction Time}) \times V}$$

B = Amount (nmole) of NADH generated between $T_{initial}$ and T_{final}.

Reaction Time = $T_{final} - T_{initial}$ (minutes)

V = sample volume (mL) added to well

G6PDH activity reported as nmole/min/mL (milliunit/mL)

One unit is the amount of enzyme that catalyzes the conversion of 1.0 μmole of glucose-6-phosphate to 6-phosphoglucono-δ-lactone and generates 1.0 μmole of NADH per minute at 37 °C.

Example:

NADH amount (B) = 5.84 nmole

First reading ($T_{initial}$) = 3 minute

Second reading (T_{final}) = 32 minutes

Sample volume (V) is 0.01 mL

Sample dilution is 1

G6PDH activity is:

$$\frac{5.84}{(32 - 3) \times 0.01} \times 1 = 20.14 \text{ milliunits/mL}$$

4

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay Not Working	Assay Buffer Ice Cold	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For fluorescence assays, use black plates with clear bottoms. For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/Higher Readings in Samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Always check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Always prepare fresh Master Reaction Mix before use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear Standard Curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the reaction mix	Prepare a Master Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to the dilutions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
		Deproteinize samples
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

LS,MF,MAM 02/13-1

©2013 Sigma-Aldrich Co. LLC. All rights reserved. SIGMA-ALDRICH is a trademark of Sigma-Aldrich Co. LLC, registered in the US and other countries. Sigma brand products are sold through Sigma-Aldrich, Inc. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see product information on the Sigma-Aldrich website at www.sigmaaldrich.com and/or on the reverse side of the invoice or packing slip.

APPENDIX D

Week	Regular Male 1 (g)	Regular Male 2 (g)	Regular Male 3 (g)	Regular Male 4 (g)
0	26.98	26.50	28.60	30.50
1	27.93	27.21	28.82	31.02
2	28.72	27.05	29.25	30.80
4	28.83	27.14	29.24	31.13
5	29.29	27.22	28.76	30.58
6	30.35	27.39	29.36	31.15
7	30.50	27.20	29.28	31.12
8	30.50	27.85	29.80	32.02
9	30.59	28.20	29.84	31.95
10	31.36	30.53*	30.53*	30.53*
11	30.88	30.74*	30.74*	30.74*
12	31.33	28.98	30.10	32.30
14	30.88	29.42	30.47	32.68

Week	High Fat Male 1 (g)	High Fat Male 2 (g)	High Fat Male 3 (g)
0	29.75	28.50	27.90
1	30.60	31.47	28.98
2	32.24	33.30	29.87
4	34.50	34.19	30.78
5	37.51	36.86	33.12
6	39.50	38.78	33.50
7	40.17	39.13	33.10
8	40.24	39.06	33.57
9	42.08	41.17	37.71
10	40.37*	40.37*	40.37*
11	39.78*	39.78*	39.78*
12	46.48	43.29	35.54
14	48.05	45.67	36.76

Raw body weights for the regular and high fat diet male mice over the 14 week feeding period. Cells marked with * indicate an average weight of the individuals in the cage. Male mice were housed together and on weeks 10 and 11, the entire cage weight was taken rather then individuals' weights.

Week	Regular Female 1 (g)	Regular Female 2 (g)	Regular Female 3 (g)
0	21.80	23.01	22.70
1	21.48	23.81	23.01
2	21.12	23.40	22.63
4	22.71	23.38	22.86
5	23.00	23.58	22.84
6	23.17	24.06	23.34
7	23.05	23.71	23.51
8	23.10	23.53	23.06
9	23.57	24.10	23.65
10	24.03	23.7	23.34
11	24.16	24.02	24.82
12	24.06	24.10	23.37
14	23.49	24.66	24.12

Week	High Fat Female 1 (g)	High Fat Female 2 (g)	High Fat Female 3 (g)	High Fat Female 4 (g)
0	21.74	21.63	21.22	21.55
1	21.41	22.41	21.30	21.23
2	22.56	23.33	22.80	21.59
4	24.20	24.69	22.30	22.43
5	24.62	24.65	22.83	22.85
6	25.37	26.30	24.56	24.38
7	25.72	27.15	23.04	23.13
8	26.06	27.91	23.38	24.07
9	27.57	29.85	23.83	23.68
10	28.04	30.02	24.3	23.59
11	29.39	31.22	23.6	24.12
12	28.50	32.93	24.84	24.24
14	31.13	34.46	26.73	24.75

Raw body weights for the regular and high fat diet female mice over the 14 week feeding period.

Mouse	Liver Weight (g)	Blood Glucose (mg/dL)	G6PDH activity (miliunits/mL)	NADPH/NADP$^+$ ratio
Regular Male 1	1.56	191	4.62	1.04
Regular Male 2	1.41	138	13.16	1.11
Regular Male 3	1.48	172	5.84	1.07
Regular Male 4	1.27	202	3.41	0.96
High Fat Male 1	3.72	266	1.13	0.70
High Fat Male 2	3.52	234	2.67	0.43
High Fat Male 3	1.55	227	5.07	0.99
Regular Female 1	0.74	138	5.87	1.67
Regular Female 2	1.14	167	5.19	1.05
Regular Female 3	1.08	148	4.59	2.18
High Fat Female 1	1.14	245	4.80	1.03
High Fat Female 2	1.29	254	2.55	1.08
High Fat Female 3	1.18	212	4.86	0.42
High Fat Female 4	1.12	198	8.06	1.31

Raw data for liver weight, blood glucose, G6PDH activity and NADPH/NADP$^+$ ratio in individual mice.

APPENDIX E

```
GET
  FILE='C:\Users\deluciac\Desktop\SPSS_1.sav'.
DATASET NAME DataSet1 WINDOW=FRONT.
T-TEST GROUPS=Sex(1 2)
  /MISSING=ANALYSIS
  /VARIABLES=FinalWeight Glucose G6PDH Ratio Liver
  /CRITERIA=CI(.95).
```

T-Test

```
[DataSet1] C:\Users\deluciac\Desktop\SPSS_1.sav
```

Group Statistics

	Sex	N	Mean	Std. Deviation	Std. Error Mean
Week14	Male	7	36.2757	7.63622	2.88622
	Female	7	27.0486	4.16168	1.57297
mg/dL	Male	7	204.2857	42.47632	16.05454
	Female	7	194.5714	45.67953	17.26524
miliunits/mL	Male	7	5.1286	3.87855	1.46595
	Female	7	5.1314	1.64651	.62232
NADPH/NADP+	Male	7	.9000	.24671	.09325
	Female	7	1.2486	.55544	.20994
Liver Weight (g)	Male	7	2.0729	1.06295	.40176
	Female	7	1.0986	.17131	.06475

Independent Samples Test

		Levene's Test for Equality of Variances	
		F	Sig.
Week14	Equal variances assumed	3.206	.099
	Equal variances not assumed		
mg/dL	Equal variances assumed	.153	.702
	Equal variances not assumed		
miliunits/mL	Equal variances assumed	1.572	.234
	Equal variances not assumed		
NADPH/NADP+	Equal variances assumed	2.325	.153
	Equal variances not assumed		
Liver Weight (g)	Equal variances assumed	18.045	.001
	Equal variances not assumed		

Independent Samples Test

		t-test for Equality of Means			
		t	df	Sig. (2-tailed)	Mean Difference
Week14	Equal variances assumed	2.807	12	.016	9.22714
	Equal variances not assumed	2.807	9.275	.020	9.22714
mg/dL	Equal variances assumed	.412	12	.688	9.71429
	Equal variances not assumed	.412	11.937	.688	9.71429
miliunits/mL	Equal variances assumed	-.002	12	.999	-.00286
	Equal variances not assumed	-.002	8.095	.999	-.00286
NADPH/NADP+	Equal variances assumed	-1.517	12	.155	-.34857
	Equal variances not assumed	-1.517	8.279	.166	-.34857
Liver Weight (g)	Equal variances assumed	2.394	12	.034	.97429
	Equal variances not assumed	2.394	6.311	.052	.97429

APPENDIX F

```
T-TEST GROUPS=Diet(1 2)
  /MISSING=ANALYSIS
  /VARIABLES=FinalWeight Glucose G6PDH Ratio Liver
  /CRITERIA=CI(.95).
```

T-Test

```
[DataSet1] C:\Users\deluciac\Desktop\SPSS_1.sav
```

Group Statistics

	Diet	N	Mean	Std. Deviation	Std. Error Mean
Week14	Regular	7	27.9600	3.76061	1.42138
	High Fat	7	35.3643	8.89798	3.36312
mg/dL	Regular	7	165.1429	25.31422	9.56787
	High Fat	7	233.7143	23.71156	8.96213
milliunits/mL	Regular	7	6.0971	3.22745	1.21986
	High Fat	7	4.1629	2.27049	.85816
NADPH/NADP+	Regular	7	1.2971	.45569	.17224
	High Fat	7	.8514	.34163	.12913
Liver Weight (g)	Regular	7	1.2400	.28148	.10639
	High Fat	7	1.9314	1.16396	.43994

Independent Samples Test

		Levene's Test for Equality of Variances	
		F	Sig.
Week14	Equal variances assumed	3.914	.071
	Equal variances not assumed		
mg/dL	Equal variances assumed	.093	.765
	Equal variances not assumed		
milliunits/mL	Equal variances assumed	.068	.799
	Equal variances not assumed		
NADPH/NADP+	Equal variances assumed	.462	.509
	Equal variances not assumed		
Liver Weight (g)	Equal variances assumed	13.366	.003
	Equal variances not assumed		

Independent Samples Test

		t-test for Equality of Means			
		t	df	Sig. (2-tailed)	Mean Difference
Week14	Equal variances assumed	-2.028	12	.065	-7.40429
	Equal variances not assumed	-2.028	8.077	.077	-7.40429
mg/dL	Equal variances assumed	-5.231	12	.000	-68.57143
	Equal variances not assumed	-5.231	11.949	.000	-68.57143
milliunits/mL	Equal variances assumed	1.297	12	.219	1.93429
	Equal variances not assumed	1.297	10.770	.222	1.93429
NADPH/NADP+	Equal variances assumed	2.071	12	.061	.44571
	Equal variances not assumed	2.071	11.125	.062	.44571
Liver Weight (g)	Equal variances assumed	-1.528	12	.153	-.69143
	Equal variances not assumed	-1.528	6.699	.172	-.69143

APPENDIX G

```
GET
  FILE='C:\Users\deluciac\Desktop\SPSS_3.sav'.
DATASET NAME DataSet2 WINDOW=FRONT.
T-TEST GROUPS=Diet(1 2)
  /MISSING=ANALYSIS
  /VARIABLES=Final Glucose G6PDH Ratio Liver
  /CRITERIA=CI(.95).
```

T-Test

```
[DataSet2] C:\Users\deluciac\Desktop\SPSS_3.sav
```

Group Statistics

	Diet	N	Mean	Std. Deviation	Std. Error Mean
Week14	Control	4	30.8625	1.35873	.67937
	High Fat	3	43.4933	5.95142	3.43606
mg/dL	Control	4	175.7500	28.05203	14.02602
	High Fat	3	242.3333	20.79263	12.00463
miliunits/mL	Control	4	6.7575	4.38210	2.19105
	High Fat	3	2.9567	1.98558	1.14638
NADPH/NADP+	Control	4	1.0450	.06351	.03175
	High Fat	3	.7067	.28006	.16169
Liver Weight (g)	Control	4	1.4300	.12302	.06151
	High Fat	3	2.9300	1.19929	.69241

Independent Samples Test

		Levene's Test for Equality of Variances	
		F	Sig.
Week14	Equal variances assumed	8.705	.032
	Equal variances not assumed		
mg/dL	Equal variances assumed	.280	.619
	Equal variances not assumed		
miliunits/mL	Equal variances assumed	1.485	.277
	Equal variances not assumed		
NADPH/NADP+	Equal variances assumed	3.297	.129
	Equal variances not assumed		
Liver Weight (g)	Equal variances assumed	16.852	.009
	Equal variances not assumed		

Independent Samples Test

		t-test for Equality of Means			
		t	df	Sig. (2-tailed)	Mean Difference
Week14	Equal variances assumed	-4.231	5	.008	-12.63083
	Equal variances not assumed	-3.606	2.157	.062	-12.63083
mg/dL	Equal variances assumed	-3.432	5	.019	-66.58333
	Equal variances not assumed	-3.607	4.989	.015	-66.58333
miliunits/mL	Equal variances assumed	1.375	5	.228	3.80083
	Equal variances not assumed	1.537	4.375	.193	3.80083
NADPH/NADP+	Equal variances assumed	2.410	5	.061	.33833
	Equal variances not assumed	2.053	2.155	.167	.33833
Liver Weight (g)	Equal variances assumed	-2.569	5	.050	-1.50000
	Equal variances not assumed	-2.158	2.032	.162	-1.50000

APPENDIX H

```
T-TEST GROUPS=Diet(1 2)
  /MISSING=ANALYSIS
  /VARIABLES=Final Glucose G6PDH Ratio Liver
  /CRITERIA=CI(.95).
```

T-Test

```
[DataSet3] C:\Users\deluciac\Desktop\SPSS_4.sav
```

Group Statistics

	Diet	N	Mean	Std. Deviation	Std. Error Mean
Week14	Control	3	24.0900	.58558	.33808
	High Fat	4	29.2675	4.36950	2.18475
mg/dL	Control	3	151.0000	14.73092	8.50490
	High Fat	4	227.2500	26.57536	13.28768
miliunits/mL	Control	3	5.2167	.64042	.36974
	High Fat	4	5.0675	2.26624	1.13312
NADPH/NADP+	Control	3	1.6333	.56589	.32672
	High Fat	4	.9600	.38009	.19004
Liver Weight (g)	Control	3	.9867	.21572	.12454
	High Fat	4	1.1825	.07588	.03794

Independent Samples Test

		Levene's Test for Equality of Variances	
		F	Sig.
Week14	Equal variances assumed	10.873	.022
	Equal variances not assumed		
mg/dL	Equal variances assumed	4.975	.076
	Equal variances not assumed		
miliunits/mL	Equal variances assumed	1.439	.284
	Equal variances not assumed		
NADPH/NADP+	Equal variances assumed	.369	.570
	Equal variances not assumed		
Liver Weight (g)	Equal variances assumed	5.947	.059
	Equal variances not assumed		

Independent Samples Test

		t-test for Equality of Means			
		t	df	Sig. (2-tailed)	Mean Difference
Week14	Equal variances assumed	-1.991	5	.103	-5.17750
	Equal variances not assumed	-2.342	3.143	.097	-5.17750
mg/dL	Equal variances assumed	-4.418	5	.007	-76.25000
	Equal variances not assumed	-4.833	4.763	.005	-76.25000
miliunits/mL	Equal variances assumed	.108	5	.918	.14917
	Equal variances not assumed	.125	3.611	.907	.14917
NADPH/NADP+	Equal variances assumed	1.902	5	.116	.67333
	Equal variances not assumed	1.781	3.328	.164	.67333
Liver Weight (g)	Equal variances assumed	-1.726	5	.145	-.19583
	Equal variances not assumed	-1.504	2.375	.252	-.19583

APPENDIX I

```
DATASET ACTIVATE DataSet1.
CORRELATIONS
  /VARIABLES=Sex Diet FinalWeight Glucose G6PDH Ratio Liver
  /PRINT=TWOTAIL NOSIG
  /MISSING=PAIRWISE.
```

Correlations

[DataSet1] C:\Users\deluciac\Desktop\SPSS_1.sav

Correlations

		Sex	Diet	Week14	mg/dL	miliunits/mL
Sex	Pearson Correlation	1	.143	-.630	-.118	.001
	Sig. (2-tailed)		.626	.016	.688	.999
	N	14	14	14	14	14
Diet	Pearson Correlation	.143	1	.505	.834	-.351
	Sig. (2-tailed)	.626		.065	.000	.219
	N	14	14	14	14	14
Week14	Pearson Correlation	-.630	.505	1	.732	-.526
	Sig. (2-tailed)	.016	.065		.003	.053
	N	14	14	14	14	14
mg/dL	Pearson Correlation	-.118	.834	.732	1	-.660
	Sig. (2-tailed)	.688	.000	.003		.010
	N	14	14	14	14	14
miliunits/mL	Pearson Correlation	.001	-.351	-.526	-.660	1
	Sig. (2-tailed)	.999	.219	.053	.010	
	N	14	14	14	14	14
NADPH/NADP+	Pearson Correlation	.401	-.513	-.609	-.618	.256
	Sig. (2-tailed)	.155	.061	.021	.019	.377
	N	14	14	14	14	14
Liver Weight (g)	Pearson Correlation	-.569	.403	.917	.553	-.452
	Sig. (2-tailed)	.034	.153	.000	.040	.105
	N	14	14	14	14	14

Correlations

		NADPH/NADP+	Liver Weight (g)
Sex	Pearson Correlation	.401	-.569
	Sig. (2-tailed)	.155	.034
	N	14	14
Diet	Pearson Correlation	-.513	.403
	Sig. (2-tailed)	.061	.153
	N	14	14
Week14	Pearson Correlation	-.609	.917
	Sig. (2-tailed)	.021	.000
	N	14	14
mg/dL	Pearson Correlation	-.618	.553
	Sig. (2-tailed)	.019	.040
	N	14	14
milliunits/mL	Pearson Correlation	.256	-.452
	Sig. (2-tailed)	.377	.105
	N	14	14
NADPH/NADP+	Pearson Correlation	1	-.555
	Sig. (2-tailed)		.040
	N	14	14
Liver Weight (g)	Pearson Correlation	-.555	1
	Sig. (2-tailed)	.040	
	N	14	14

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).