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By

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This thesis has been examined and approved by the following members of the student's committee.

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ABSTRACT

High Fat Diet Increased Serum Glutamate Dehydrogenase more than Chronic Acetaminophen Dosing in Female Mice

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This laboratory study examined the effects of acetaminophen overdose in normal fed and high-fat fed female mice. Forty female mice were placed on normal and high-fat diets at 4 weeks old. When the mice were significantly different in weight (between 6 and 9 months old), half the mice were dosed with acetaminophen. These mice were daily given an overdose of acetaminophen for 14 days. The dose used was 300 mg/kg mouse (LD_{50} = 338 mg/kg). The control group was given 10 µl water/g mouse.

Levels of serum glutamate dehydrogenase (GDH) were measured to indicate liver damage. GDH is released from liver mitochondria indicating mitochondrial damage and toxicity. Based on a two-way ANOVA, the mean GDH levels were significantly higher in the high-fat diet mice groups (p <0.001). This suggests damage to liver mitochondria due to high-fat diet alone.

Liver weights of all female mice groups were documented and statistically analyzed and showed significant difference between diet groups (p <0.001) and diet*treatment groups (p = 0.026). Visible fat content in all livers was also analyzed and showed significant difference between diet groups (p <0.001) and treatment (acetaminophen or control) groups (p = 0.001).

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Chapter I

INTRODUCTION

Acetaminophen (APAP) overdose is the leading cause of calls to the Poison Control Center in the United States. Overdose of this drug also accounts for 50% of all liver failure in the U.S. APAP overdose accounts for over 55,000 hospital visits, 2,600 emergency room visits, and over 450 deaths About half of these deaths are the result of intentional overdose while the other half is the result of accidental overdose (Lee, 2004).

Other factors, such as obesity, play a significant role in increasing the likelihood of liver damage while taking acetaminophen. This is due to the damaging effects of acetaminophen on mitochondria in a fatty liver (Aubert and Delannoy, 2012; Zimmerman, 1995). The combination of acetaminophen overdose and fatty liver may pose unintentional hazards to the health of its users.

The known disease associated with fat accumulation in the liver is called nonalcoholic fatty liver disease (NAFLD) (Duvnjak, 2007). Fatty liver disease has been seen in 70-80% of obese individuals. NAFLD may lead to insulin resistance, cirrhosis or scaring of the liver, metabolic syndrome and type 2 diabetes (Farrell, 2006; Ozturk, 2014).

Obesity has risen greatly in adolescents over the past several years. Obesity in children aging from 6-11 years old increased from 7% in 1980 to 18% in 2010

(Ogden et al., 2010). In 2010, 1 out of 3 children was obese and thought to have NAFLD (Ogden et al., 2010; National Center for Health Statistics, 2011).

The increase rise in obesity has shown affect the age of onset of puberty in both males and females (Wagner et al., 2012). When compared to males, it is evidently clear that female adolescents reach puberty earlier in response to obesity (Ozturk, 2014). Liver failure from overdose of acetaminophen has also specifically affected women (Ferguson et al., 1977). Therefore, the danger of acetaminophen and obesity toxicity may be especially harmful to females and their liver function.

Specifically, the liver plays a role in puberty and can greatly alter puberty onset if damaged. Eren and colleagues (2009) found that liver damage can cause precocious puberty (early puberty) in females. Liver damage can affect secretion of testosterone, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), all hormones necessary for puberty to occur (Eren et al., 2009).

Most acetaminophen is given orally. For example, Children's Tylenol® gives its recommended acetaminophen dose based on weight. As a result, young, obese females being administered acetaminophen at higher dosages may be at greater risk for liver damage. Table 1 shows the dosage chart for Children's Tylenol®, also known as acetaminophen (Tylenol.com - Children's Dosage Guide, 2013). The children's ages are associated with their average weights. Concerned parents may accidentally provide an overdose of acetaminophen for an overweight child.

Child's Weight *Child's Age*	Children's Tylenol® Suspension Liquid
24-35 pounds *2-3 years*	5 mL
36-47 pounds *4-5 years*	7.5 mL
48-59 pounds *6-8 years*	10 mL
60-71 pounds *9-10 years*	12.5 mL
72-95 pounds *11 years*	15 mL

Table 1*. Children's Tylenol® dosage chart.*

The primary purpose of this study was to determine the effects of acetaminophen-induced liver toxicity and obesity-induced liver toxicity. A main hypothesis was formed at the onset of this study: the effects of acetaminophen dosages in the high fat-fed mice will show greater indications of liver damage than mice on a normal fat-fed diet. This is because 1) the liver will see a higher dose in the obese mice which will cause liver damage and 2) the liver cells will become fatty from the high-fat diet which will also harm the liver. A secondary purpose of this study was to determine the relationship between diet-induced liver damage and drug-induced liver damage.

The hypothesis was tested in a laboratory setting with C57/BL6 strain wild-type female mice. Adolescent mice were used to assess how their higher fat content affected damage from acetaminophen. Acetaminophen and fat-induced liver

damage was measured based on glutamate dehydrogenase (GDH) activity levels. Livers were also examined for fat content by weight and visual analysis.

Through these laboratory analyses, it was found that fat toxicity had more damaging effects on liver mitochondria of female mice than chronic acetaminophen dosing. Levels of GDH were significantly higher only in female mice given a high fat diet.

Liver weights confirmed liver hypertrophy due to fat intake, but also indicated further hypertrophy in high-fat fed female mice given acetaminophen. This reflects additional damage to the liver by acetaminophen not reflected in mitochondrial damage of GDH.

Visible fat content also confirmed the increased liver accumulation of fat in the high fat diet fed mice. However, acetaminophen increased visible fat content especially evident in the high fat diet. In this case, this data suggests that acetaminophen damage to structures other than the mitochondria may be responsible for fat accumulation.

Chapter II

LITERATURE REVIEW

Background

Acetaminophen is considered a safe, "over the counter" analgesic used to relieve pain in children and adults. However, acetaminophen is known to cause liver injury with overdose (Shivbalan et al., 2010) as well as with a maximum recommended dosage (Michaut and Moreau, 2014). Misuse of this drug, both intentional and by accident, is the leading cause of acute liver failure in the United States (Myers et al., 2008).

Acetaminophen-induced liver toxicity is much more likely in people with alcohol problems, other underlying liver disease, and those having an unintentional overdose of the drug (Myers et al., 2008). The severity of acetaminophen-induced liver injury may also be enhanced with nonalcoholic fatty liver disease (NAFLD), which refers to liver damage associated with obesity (Michaut and Moreau, 2014).

Specifically as our nation's children, and especially females, grow up obese, their risk of drug overdose associated with NAFLD may have profound damaging effects on their livers. The mechanisms involved with obesity-induced liver toxicity and acetaminophen-induced liver toxicity, as associated with female adolescents, will be described in subsequent sections.

Obesity-induced Steatosis

Mitochondrial Dysfunction. With the prevalence of obesity rising in young adults, taking acetaminophen may result in higher doses to achieve pain relief, even though the dosage will be the same based on weight. Fat can accumulate in liver cells and have damaging effects similar to those of acetaminophen and its toxic metabolites.

Specifically, non-alcoholic fatty liver disease (NAFLD) has increased over the last 30 years (Ozturk, 2014) and is now viewed as the most common form of liver disease in children and adolescents (Schwimmer, 2006). This disease is associated with type 2 diabetes, cardiovascular disease, and liver cancer (Barshop, 2008).

NAFLD includes many diseases that can range from simple steatosis (hepatic fat accumulation) to non-alcoholic steatohepatitis (NASH). NASH is shown by lipid accumulation associated with liver cell inflammation, injury, and fibrosis. NASH may progress to cirrhosis and end-state liver disease. Alkhouri and colleagues (2014) showed that children with NASH may be at an increased risk for cardiovascular diseases as well.

Steatosis is specifically fat accumulation in liver cells. The accumulation of triglycerides in hepatocytes is known to be the first severe attack in the progress of liver disease (Berlanga, Alba et al., 2014). This fat accumulation can inhibit β-oxidation of fatty acids, causing cell death and increased fatty acid synthesis. Increased fatty acid synthesis is due to the increased regulation of

lipogenic pathways in the liver, which are pathways which store energy as fat (Sudheer and King, 2008; Eaton and Zaitoun, 1996).

Ji and colleagues (2014) examined how overfeeding during sensitive developmental periods increases the risk of obesity later in life. Their group looked at the relationship among postnatal nutrition, lipid metabolism, and NAFLD progression during development. They used litters of mice pups and put them on a high-fat diet beginning at 21 days old. According to their study, at 16 weeks old, the high-fat groups showed obesity and insulin resistance. They concluded that overfeeding during development contributed to NAFLD through the up-regulation of hepatic lipogenesis, which is the accumulation of fat storage in liver cells (Ji et al., 2014).

In summary, fat accumulation will begin with steatosis and ultimately result in cirrhosis (scarring of liver tissue) and liver cancer if not treated effectively. The progression of liver damage was outlined by Reddy and colleagues (2006). They showed with models how fat deposition causes liver enlargement. After liver enlargement, scar tissues forms in the liver causing fibrosis and liver injury. Liver fibrosis may lead to cirrhosis, which involves the physical hardening of liver tissue, causing it to become completely dysfunctional (Reddy, 2006).

The process of liver steatosis begins with the accumulation of triglycerides in hepatocytes. Fat cells, or adipocytes, fill up the hepatocytes so that the adipocytes fill almost the entire liver cell. This can be seen as macrovesicular (large vesicle) or microvesicular (small vesicle) fat droplets in hepatocytes (Reddy, 2006). Fatty acid overload in hepatocytes induces microsomal cytochrome P-450 (CYP2E1) and fatty

acid oxidation systems which create reactive oxygen species (ROS). These ROS result in oxidative stress of mitochondria leading to their destruction (Rao, 2004). Oxidative stress causes the release of several cytokines including TNF-α by hepatocytes (Day, 1998).

Reactive oxygen species activate cells that begin to create cirrhosis. Lipid peroxidation products and proteins modified by ROS cause an inflammatory response (Reddy, 2006). These destructive autoimmune responses toward hepatocytes play a critical role in liver injury by adipocytes. Figure 1 on the following page shows a representation of cirrhosis damage by ROS (Parsian, 2011).

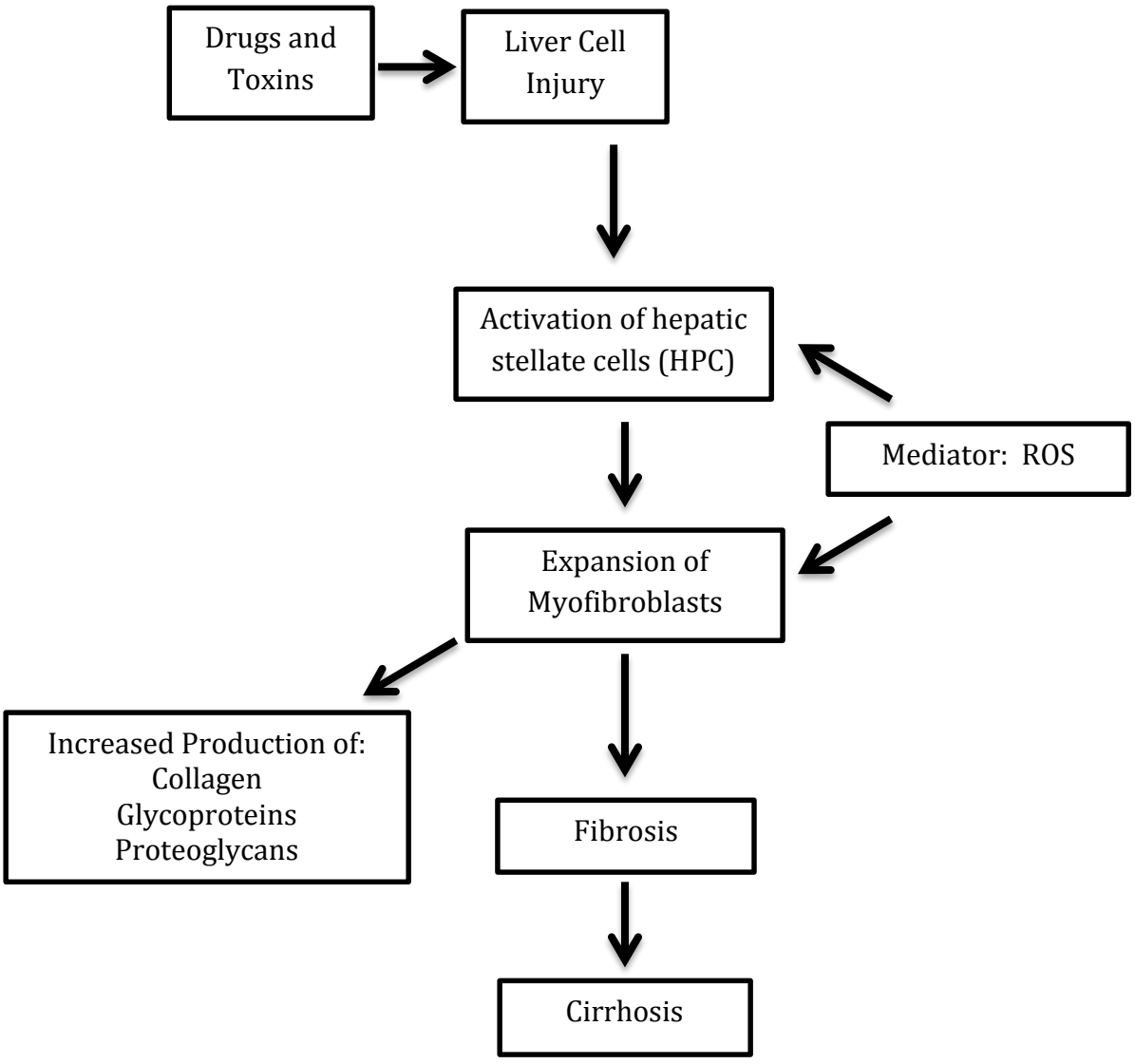


Figure 1. *Pathway of cirrhosis by Reactive Oxygen Species (ROS) (Parsian, 2011).*

Female vs. Male. In 2010, about 1 in 5,000 children experienced early puberty. Research has shown that an earlier age of puberty corresponds to increased incidences of obesity. For example, in 1965 about 5% of children were obese. In 2010, 18% of children were obese (Ogden et al., 2010).

Obesity may have a different effect on adolescent males. Boys who are obese may enter puberty at a later stage. Vandewalle and colleagues (2014) studied sex steroid levels in obese adolescent males. They found that testosterone levels were lower in obese males at puberty, but skeletal maturation and estradiol were increased in obese boys at the beginning of puberty (Vandewalle et al., 2014). This suggests that estradiol contributes to the advancement of skeletal maturation, but the low levels of testosterone may delay puberty in adolescent boys.

Research has also shown that obesity may play a role in the age a female enters puberty (Ozturk, 2014; Addo et al., 2014). In girls who enter puberty earlier than 8 years of age, it is considered precocious puberty. Dr. Addo and colleagues suggest a link between early puberty in girls and obesity. Their research looked at hormone level concentrations in obese females entering puberty. They found that preadolescent weight gain lowered the age of Luteinizing Hormone (LH) onset. LH is needed for puberty to occur, and higher levels of LH were seen earlier in obese females indicating earlier puberty onset.

Dr. Addo and colleagues (2014) also showed that the link between obesity and early puberty was not only seen in white Caucasian females, but in female girls from all ethnic groups. Using the rise of LH as their maker, their research showed

that the age of puberty onset among obese females was first seen in non-Hispanic black girls at 10.08 years, followed by Mexican-American girls at 10.64 years, and at 10.66 years for non-Hispanic white.

Vannucci and colleagues researched eating disorders including binge eating during puberty. Their research suggested that puberty is a critical risk period when binge eating behaviors found in boys and girls may attribute to weight gain, body shape concerns, and more frequent food consumption (Vannucci et al., 2014).

In summary, obesity has obvious effects on females entering puberty early. Studies have shown how overeating can contribute to obesity and detrimentally affect females during important developmental phases of their lives. Growing up obese may even affect their ability to reproduce as adults (Lai et al., 2014). Pre-puberty females growing up obese may enter puberty earlier, and they also may have compromised liver function because of increased fat storage in their liver cells.

Acetaminophen-induced Hepatotoxicity

Mitochondrial Dysfunction. As previously stated, acetaminophen is mainly used as an analgesic medicine for pain and fever relief (Casarett et al., 1980; McGill et al., 2012). When taken orally, most of acetaminophen is converted to non-toxic metabolites by Phase-II drug metabolism reactions through conjugation with sulfate and glucuronide (Casarett et al., 1980). However, at high doses, acetaminophen produces a toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI) by means of cytochrome P-450 enzymes (specifically CYP2E1) in the liver (Aubert and Delannoy, 2012). A small percentage of NAPQ1 is detoxified by glutathione (GSH). Glutathione is a tripeptide antioxidant that protects cells against oxidative damage from reactive oxygen species, and it also plays a crucial role in detoxifying various drugs including acetaminophen (Botta et al., 2009).

Additional NAPQ1 from high doses of APAP depletes the liver of GSH (Ben-Shachar and Yifei, 2012). The depletion of GSH allows the toxic metabolite, NAPQ1, to further harm the liver by damaging the mitochondria (Jaeschke and Gores, 2002). In fact, one method of treatment for acetminophen overdose is the administration of N-acetylcysteine (NAC). NAC increases GSH synthesis in the liver, helping to eliminate excess NAPQ1 (Shayiq et al., 1999).

Figure 2 shows the pathways of acetaminophen metabolism in the liver (Marshall and Bangert, 1995). Acetaminophen is safely conjugated through glucuronidation and sulfation to lead to non-toxic metabolites. Glucuronidation is the addition of a glucuronic acid to a substrate group. This addition makes the

compound more water-soluble and able to be eliminated by the body. Sulfation is the addition of a sulfate to a substrate group. Sulfation makes the compound less toxic as well.

NAPQ1 can be safely detoxified with GSH when there is no acetaminophen overdose. In situations of acetaminophen overdose, GSH is depleted, and NAPQ1 continues to react with mitochondrial proteins and harm the liver as it overwhelms the acetaminophen metabolism system (Marshall and Bangert, 1995).

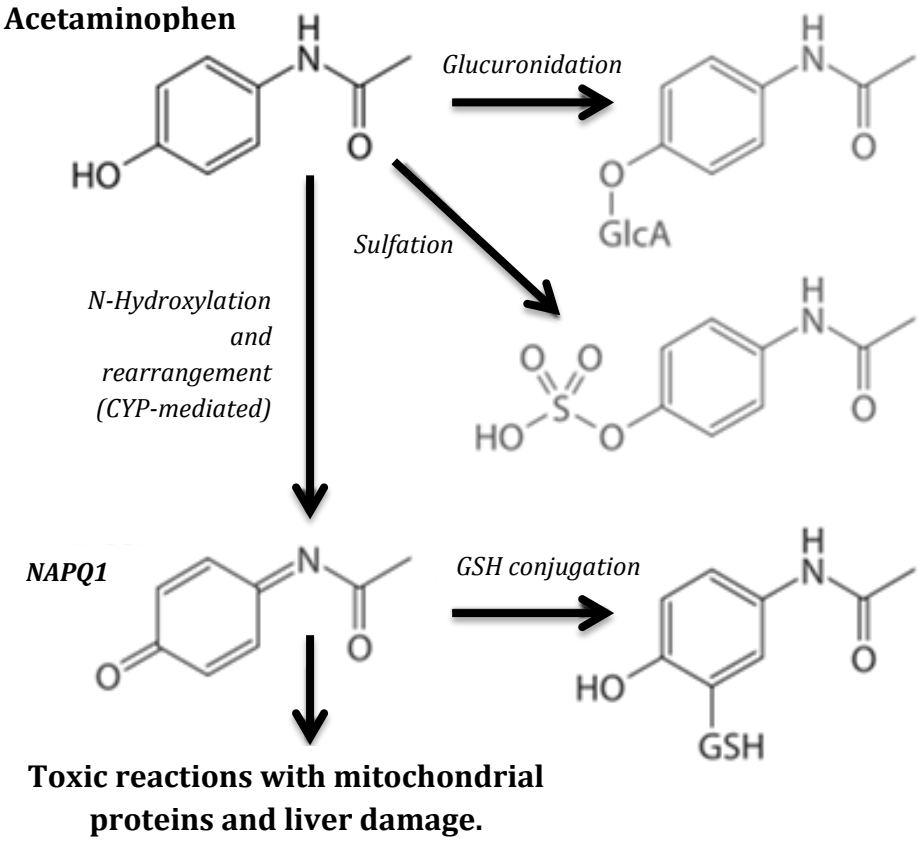


Figure 2. *Pathways of Acetaminophen Metabolism* (Marshall and Bangert, 1995).

The toxic metabolite, NAPQ1, has been shown to bind to mitochondrial proteins which lead to mitochondrial oxidative stress and dysfunction (Bait et al., 2006; Jaeschke, H. 1990; Jaeschke et al., 2012). The binding of NAPQ1 causes the inner matrix of the mitochondria to swell and pore opening in the mitochondrial membrane. This swelling leads to membrane lysis (Placke et al., 1987). Also, pore opening results in the destruction of membrane potential and loss of ATP synthesis, which will eventually lead to necrosis of the hepatocytes (Kon et al., 2004).

The increased permeability and lysis of the mitochondrial membrane releases enzymes from the inner matrix such as glutamate dehydrogenase (GDH) (Zhang et al., 2010). GDH is a mitochondrial enzyme needed for urea synthesis in the liver (Placke et al., 1987). Elevated levels of GDH indicate mitochondrial dysfunction and damage due to NAPQ1 (Antoine et al., 2010).

The mitochondria of a cell are responsible for making ATP to generate energy for the processes of a cell. Specifically this process of generating ATP is called β-oxidation. NAPQ1 not only disrupts the membrane of the mitochondria to cause lysis, but directly inhibits β-oxidation by damaging the mitochondria itself (McGill et al., 2012; Russmann et al, 2009). A decrease in cellular ATP will directly cause necrosis, cell death, of hepatocytes (liver cells).

Another level of liver damage is seen by elevated levels of tumor necrosis factor—alpha (TNF-alpha) (Jaeschke and Gores, 2002). TNF-alpha is released as an immune response in an attempt to kill cells when they recognize imminent damage.

For example, when NAPQ1 is formed, hepatocytes will release TNF-alpha in an effort to kill the effected cells (Antoine et al., 2010).

Liver damage can also be seen through sampling biomarkers in the blood serum. The following biochemical makers are often elevated in liver damage: alanine transferase (ALT), alkaline phosphatase (ALP) and bilirubin (Brzeźnicka, 1989; Nesseler, 2012). This study recognizes these biomarkers are elevated in hepatotoxicity, but this project will focus on liver damage through GDH levels and weight and visible fat analysis of fatty liver.

Female vs. Male. Acetaminophen-induced liver damage from APAP overdose causes different metabolic changes to occur between male and female mice during its detoxification (Mohar et al., 2014). Many studies indicate that male mice show greater sensitivity to acetaminophen-induced liver injury than females. Several of those studies will be discussed here.

Mohar and colleagues aimed to identify acetaminophen metabolites and/or protein adducts associated with gender-specific metabolic pathways of acetaminophen toxicity (Mohar et al., 2014). Their research team administered acetaminophen at 300 mg/kg to both male and female mice and measured serum alanine transferase (ALT) activity at various hours after administration. While females showed eventual liver damage comparable with male mice, it was the male mice that showed the marked elevation in ALT after 6 hours.

As previously stated, synthesis of GSH helps detoxify NAPQ1 (the toxic metabolite of acetaminophen) safely from the liver. An enzyme known as

glutamate-cysteine ligase is the rate-limiting step in GSH synthesis (Botta et al, 2009). Botta and colleagues showed male mice induced to overexpression of glutamate-cysteine ligase to be more resistant to acetaminophen-induced liver injury. This is because an overexpression of glutamate-cysteine ligase would increase GSH synthesis. Since people vary in their glutamate-cysteine ligase activity, this enzyme may help in determining the sensitivity of humans to acetaminophen induced liver in jury. In summary, Botta and colleagues discovered a clear inverse relationship between glutamate-cysteine ligase activity and serum ALT levels after acetaminophen treatment in male mice. They showed that the more activity of glutamate-cysteine ligase increased synthesis of GSH, helping to eliminate NAPQ1 from the liver and thus decreasing serum ALT levels (a clear indication of liver damage). This relationship was not shown in female mice, suggesting that male mice are more sensitive to acetaminophen-induced liver damage (Botta et al., 2009).

McConnachie and collegues (2007) showed that male mice demonstrated greater acetaminophen-induced hepatotoxicity than female mice. Their group also looked at glutamate-cysteine ligase as the rate limiting step in GSH biosynthesis. McConnachie showed that treatment with N-acetylcysteine (NAC), which also helps synthesize GSH, lessened the effects of acetaminophen overdose. Ultimately further studies must be done to understand the roles of gender and glutamate-cysteine ligase activity in acetaminophen-induced liver toxicity in mice (McConnachie et al., 2007).

Furthermore, acetaminophen overdose not only harms the liver, but also the kidneys. Not surprisingly, Hu and colleagues (1993) found increased renal necrosis induced by APAP in male mice compared to female mice (Hu et al., 1993). Acetaminophen and its metabolism in the body to toxic metabolites has obvious effects on various parts of the body.

It should be noted that acetaminophen overdose causes damage in both male and female mice, but the current research shows males being more sensitive to its metabolic pathways and apparent toxicity. Because current research focuses on male mice, female mice and their acetaminophen sensitively may be overlooked. Further research must be done in order to find the differences of acetaminophen-induced liver toxicity in both males and females.

Justification for this Study

The intent of this study was to recognize the need for further analysis concerning liver damage due to acetaminophen overdose in obese female adolescents. Both males and females show obvious liver damage from acetaminophen overdose. Current research shows male mice being more sensitive to the toxic effects of acetaminophen. Adolescents, especially female, may be overlooked in scientific studies because of these results. Because of an obese female's early entry into puberty, the female gender should be further studied in regards to obesity and acetaminophen toxicity.

Countless obesity studies and experiments are being carried out in labs. They are being done for good reason because of the 33% of Americans who are obese and the damaging affects to all parts of the body due to obesity, not just the liver. Obesity may be especially damaging to developing bodies in adolescents (Ji et al., 2014).

This study looks specifically at liver damage in regards to drug overdose. Acetaminophen overdose is the leading cause of acute liver failure, both by intentional usage and by accident (Myers et al., 2008). One can understand the potential for serious liver damage when an obese female adolescent overdoses on acetaminophen.

Chapter III

MATERIALS AND METHODS

The basic experimental design involved setting up 2 groups of mice, half on a normal diet (ND) and half on a high-fat diet (HFD). Half of each diet group was then dosed with acetaminophen (APAP) while the other control group was dosed with water. Blood serum levels were analyzed for glutamate dehydrogenase (GDH) activity. Livers were weighed for fatty content as well as analyzed for visible fat analysis. The mice used in this study received Institutional Animal Care and Use Committee approval on October 3, 2012 (IACUC approval Number: #12-06).

Animals and Experimental Protocols

Forty (40), C57/BL6 strain wild-type female mice were used in this study. The mice were taken from a breeding colony in which the males were used for a separate obesity study. All female mice were bred and housed locally in the Animal Care Facility at Minnesota State University, Mankato. They were housed at standard laboratory conditions (22°C for a 12-hour light and 12-hour dark cycle) with free access to rodent feed and water.

After four weeks of normal feeding, half (20) of the mice were placed on a high-fat (11%) diet while the other half (20) remained on the normal (4%) diet. The mice were fed and carefully observed for 30-40 weeks.

The mice were weighed daily, and these weights (in grams) were documented until the mice in the two groups (normal and high-fat diet) were significantly different in weight. Significant difference in weight between the two groups was seen between 30-40 weeks from time of birth.

The female mice were then randomly divided into 2 control groups and 2 APAP-administered groups. The experimental design setup is shown in Figure 3.

Diet	Control	Experimental
High-Fat (11%)	Dosed with water (N=10)	Dosed with APAP (N=10)
Normal (4%)	Dosed with water (N=10)	Dosed with APAP (N=10)

Figure 3. *Experimental design setup.*

When the normal and high-fat fed mice were significantly different in weight, acetaminophen (Sigma-Aldrich) was daily administered to half of each group. APAP was administered for 14 days to half (10) of the normal fed and half (10) of the high-fat fed mice.

The dosages of APAP were given orally to each mouse by a calibrated 200-1000 µl pipette in 300 mg acetaminophen/kg mouse (LD_{50} = 338 mg/kg). This dosage was selected from examining past studies done with mice and dosing them with acetaminophen (Ayoub, 2004; Brzeźnicka, 1989). The volume of water was 10 ml/kg per mouse. 300 mg acetaminophen/10 ml water = 30 mg acetaminophen/ml.

The mice were dosed as 10 µl per gram mouse with weights between 20-50 g/mouse = 200-500 µl APAP administered per mouse.

The dose was very close to a lethal dose, so the mice were carefully monitored based on their appearance, movement, and food and water intake. Specifically, any mice that showed evidence of lack of grooming, and no movement when prodded with a finger were examined carefully. If any mouse became sick (eyes nearly shut but not asleep, labored breathing, few movements when prodded, and/or fur clearly not groomed), they were to be euthanized by CO_2. Fortunately, the mice in this design did not become sick during the dosing procedure.

At the end of the dosing period (14 days), the mice were euthanized by CO_2. They were then dissected, and each specific mouse's blood was collected for analysis. Livers were photographed, weighed, and split in two. Half of each liver was frozen for future analysis and the other half was saved in 10% formalin solution.

Timeline

Half of the mice began their high-fat diet at 4 weeks old. This is comparable to a 3 year old human. The mice were dosed with Tylenol around 30-40 weeks when significant difference was achieved between groups. This corresponds to a 20-36 year old human, or mature/middle age adult. The mice were then euthanized soon after. These ages correspond to the time a young human may consistently begin eating real food (3 years old) through the time of their major developmental

changes: puberty and sexual maturity. Figures 4 and 5 below shows the comparison between mouse and human ages (Flurkey, 2007). The experimental design of this project was developed to show the detrimental effects of acetaminophen and obesity in developing females, ages 3 to middle age.

Mice Life Phase	Time period (human female age)
Sexually mature	35 days (10-14 years)
Mature adult	3-6 months (18-35 years)
Middle age	10-15 months (36-55 years)
Old age	18-24 months (>55 years)

Figure 4. *Comparison of mice and human ages (Flurkey, 2007).*

Experimental Design	Human age
High-fat diet began at 4 weeks old	Preadolescent (3 years old)
Mice dosed between 7-10 months	Late mature adult/early middle age (30-40 years old)

Figure 5. *Ages of experimental design setup.*

Extraction of Blood Serum for Liver Enzymes

Each mouse's blood was extracted after euthanization. Blood was collected from the heart (superior vena cava) and surrounding area where it pooled after death. Total blood collection was about 0.5 mL. Whole blood was collected in tubes, and the blood was spun down at 1500 rpm for 10 minutes (Eppendorf Centrifuge 5415 D). Serum was then removed with a pipet, and was frozen for future analysis of GDH activity.

Liver Storage and Data Collection

After blood was collected, mice livers were removed, photographed, weighed, and split in halves. The liver's visible fat content was determined on a scale of 1, 2, or 3 (no fat, some fat, mostly fat). Any changes in color were noted as well (yellowing instead of brown). This was done partially blinded, meaning the livers were taken from known diet groups, but specific mice were not known and whether they had been dosed with acetaminophen or water.

Half of the liver was fixed in formalin (10%) for a separate project in histopathological evaluation. The other half was frozen at -20 degrees C and held for future biochemical analysis.

Handling of Mice Post-analysis

All mice were euthanized at the end of the 14 days of dosing with carbon dioxide (CO_2) in a controlled environment. After the mice were dissected, livers and blood were extracted, and the carcasses were then frozen.

Mice that were not given acetaminophen were disposed of by freezing and calling Environmental Health for disposal of non-hazardous animals. Those mice given the acetaminophen were properly labeled with the accurate amount of drug dose given. This labeling also included the number of days the mice received the drug, which allowed Environmental Health to decide whether they should be considered a hazard needing special handling. The Environmental Health team came and collected the control mice and those mice dosed with acetaminophen.

Protocols and Statistical Analysis

A glutamate dehydrogenase (GDH) activity assay kit (BioVision, Catalog #K729-100) was used to test for elevated levels of GDH in the serum. A microspectrometer (Thermo Electron Corporation, Multiskan Spectrum) took four 450 nm readings at 3 minutes, 30 minutes, 60 minutes, and 90 minutes. This occurred at an incubation temperature of 37°C. A NADH Standard curve was established to calculate GDH activity using the following equation:

$$GDH\ Activity = \frac{B}{(T \times V)} \times Sample\ Dilution\ Factory = \frac{\frac{nmol}{min}}{ml} = \frac{mU}{ml}$$

Where: ***B*** is the NADH amount from Standard Curve (in nmol).

T is the time incubated (in min).

V is the sample volume added into the reaction well (in nmol).

All statistical analyses were made using IBM SPSS Statistics 22. Unless otherwise noted, statistical significance was accepted for any parameter where $p \leq 0.05$.

The number of mice in each treatment group (N) decreased in the GDH activity results. This was because the microspectrometer froze after 4 hours, losing the data of several of the mice. Also, many wells did not show readings of GDH activity. However, the number of mice (N) for each group in regards to liver weight and fat visibility were the same (55 in total).

Chapter IV

RESULTS

Measurement of GDH Activity

A two-way analysis of variance (ANOVA) revealed statistical difference between groups. The two-way ANOVA p values were <0.001 for variable diet, 0.103 for treatment (acetaminophen or control) and 0.310 for diet*treatment. Table 2 shows the mean GDH activity level (in mU/ml) for all four groups, along with standard deviation and the number of mice (N) for each group. A higher GDH activity level corresponds to a higher degree of liver damage.

Diet	**Treatment**	**GDH Activity, mU/ml serum (Mean±SD [N])**
4% Fat	None	1.5 ± 1.0 (4)
4% Fat	300mg/APAP/kg	2.6 ± 0.9 (5)
11% Fat	None	4.1 ± 0.9 (8)*
11% Fat	300mg/APAP/kg	4.3 ± 0.7 (6)*

Table 2. *GDH Activity Levels of treatment groups.*

*Indicates significant difference based on a two-way ANOVA (p <0.001 for diet), followed by a significant difference from 4% fat control based on LSD posthoc test ($p \leq 0.05$).

Least Significant Difference (LSD) Multiple Comparisons—GDH Activity

Statistical difference ($p \leq 0.05$) was seen between diet groups regarding GDH activity levels ($p < 0.001$). This led to a LSD posthoc test showing differences between diet groups.

Significant difference was seen between the 4% and 11% diet groups dosed with water ($p < 0.001$). Significant difference was also seen between the 4% and 11% diet groups dosed with acetaminophen ($p = 0.004$). This data reveals damage done to the liver due to high fat diet alone.

Figure 6 shows a bar graph representation of mean GDH activity levels between all groups. The letters above the graphs show significant difference ($p \leq 0.05$). The error bars are within one standard deviation. This bar graph representation shows the damage to the liver due to fat toxicity alone. Table 3 shows GDH activities levels of all 23 mice.

Figure 6. *Bar Graph of Mean GDH Activity.*

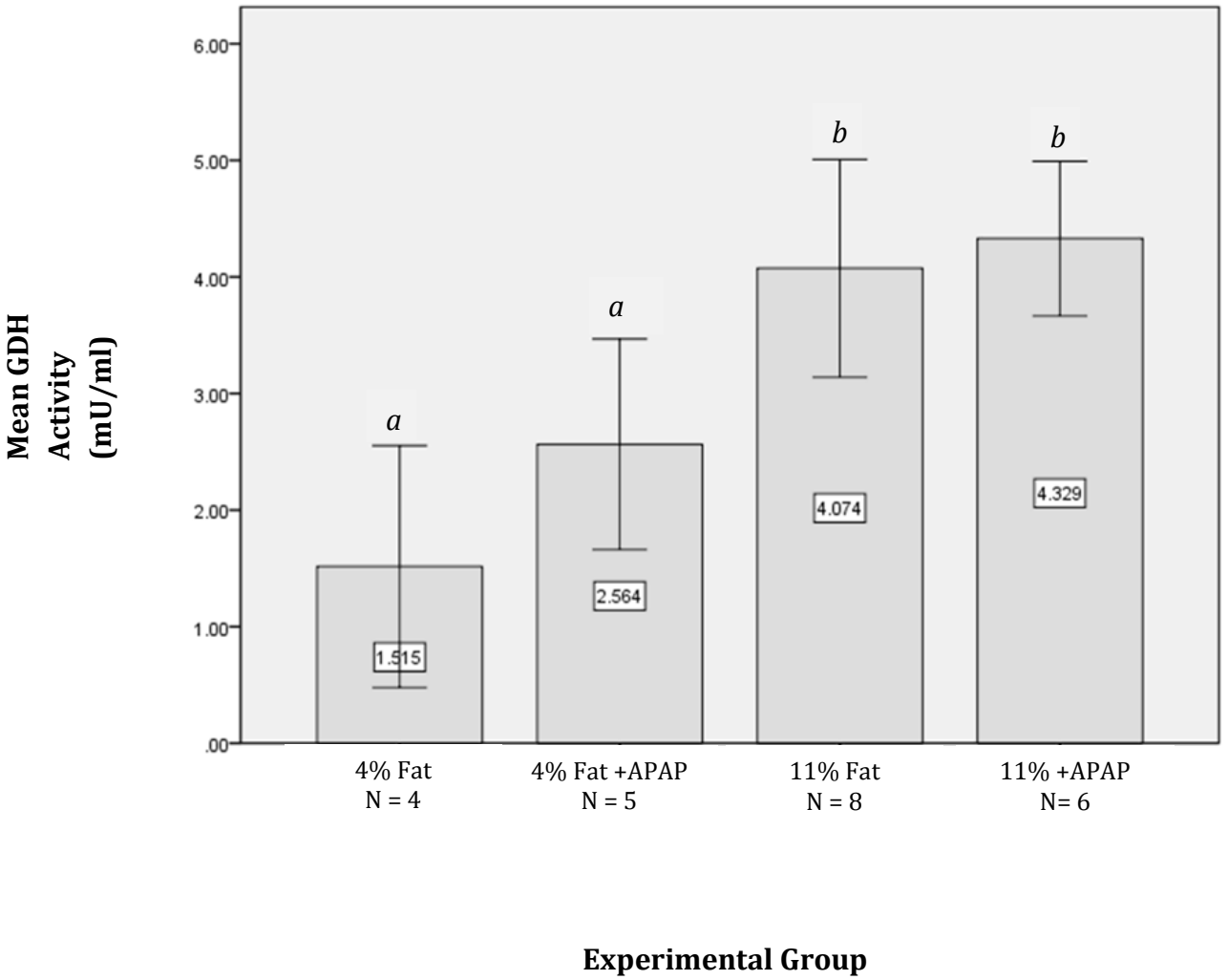


Table 3. *GDH Activity Levels (mU/ml) of all 23 mice.*

Normal -APAP	***Normal +APAP***	***High Fat-APAP***	***High Fat + APAP***
0.95739454	3.985120792	5.843517106	5.201545501
0.367926794	2.87117698	4.10633287	3.566746173
2.640560239	1.922861595	4.600608543	3.738020826
2.096108212	2.298972945	4.697737161	5.021282775
	1.742616247	3.502247025	4.285562328
		3.479635549	4.160252484
		3.047995956	
		3.310211657	

Liver Analysis of Weight and Fat Visibility

A two-way analysis of variance (ANOVA) revealed statistical difference between groups regarding liver weight and fat visibility. The mouse livers were analyzed by weight (in grams) and by a visible weight analysis based on a scale of 1, 2, or 3 (no visible fat content, some fat, or mostly fatty liver).

The two-way ANOVA *p* values for liver weight were <0.001 for variable diet, 0.066 for treatment (acetaminophen or control) and 0.026 for diet*treatment interaction.

The following two tables show the mean and standard deviation of the liver weight and visible fat content. They also show the number of mice in each experimental group.

Table 4 shows the comparisons of liver weights between groups of mice. Significant difference in liver weight was seen between the 4% fat diet and 11% fat diet groups of mice (p <0.001), as well as for the diet*treatment interaction (p = 0.026). Table 4 shows that mice fed a high fat diet had heavier livers and that acetaminophen increased liver weight significantly in the high fat-fed mice only (diet*treatment interaction).

Diet	**Treatment**	**Mean Liver Weight, grams (Mean±SD [N])**
4% Fat	None	1.3 ± 0.0 (10)
4% Fat	300mg/APAP/kg	1.3 ± 0.0 (15)
11% Fat	None	1.6 ± 0.2 (10)*
11% Fat	300mg/APAP/kg	1.8 ± 0.1 (20)*+

Table 4. *Mean and Standard Deviation of Liver Weights.*

*Indicates significant difference based on two-way ANOVA (p <0.001 for diet,) followed by a significant difference from 4% fat control based on LSD posthoc test ($p \leq 0.05$).

+ Indicates significant difference (p = 0.026 for diet*treatment interaction) from the 11% fat diet (no APAP) group.

The two-way ANOVA *p* values for visible fat content of livers were <0.001 for variable diet, 0.001 for treatment (acetaminophen or control) and 0.079 for diet*treatment interaction. Table 5 shows the comparisons of visible fat content in livers between groups of mice. Significant difference in visible liver fat was seen between the 4% fat diet and 11% fat diet groups of mice (p <0.001), as well as for treatment of acetaminophen or water (p = 0.001). This shows that mice on a high-fat diet had higher visible fat content in the liver. Table 5 also indicates acetaminophen increased the visible fat content of the liver, which is especially evident for the high fat group.

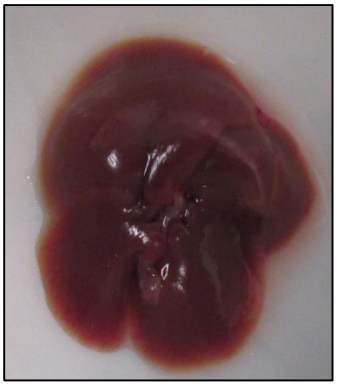
Diet	**Treatment**	**Mean Fat Visibility (Mean±SD [N])**
4% Fat	None	1.0 ± 0.0 (10)
4% Fat	300mg/APAP/kg	1.2 ± 0.4 (15) ⁺
11% Fat	None	1.6 ± 0.5 (10)*
11% Fat	300mg/APAP/kg	2.2 ± 0.4 (20)* ⁺

Table 5. *Mean and Standard Deviation of Fat Visibility.*

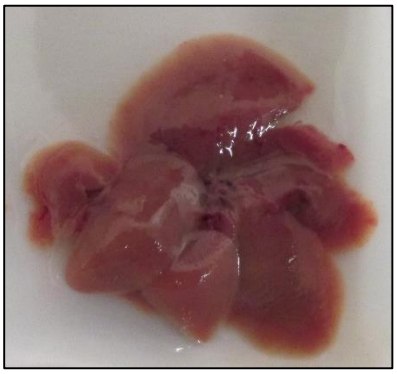
*Indicates significant difference based on two-way ANOVA (p <0.001 for diet,), followed by a significant difference from 4% fat control based on LSD posthoc test ($p \leq 0.05$).

⁺Indicates difference (p = 0.001) from no acetaminophen (treatment effect).

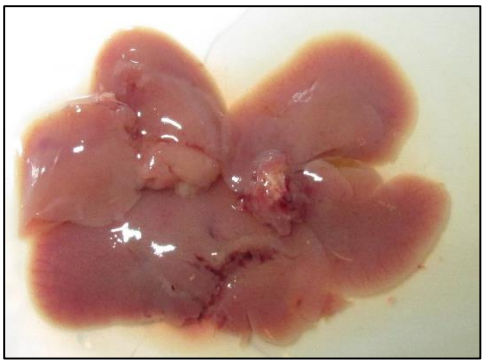
Figure 7. *Examples of liver visible fat content.*



No Fat (1)



Some Fat (2)



Mostly Fat (3)

Least Significant Difference (LSD) Multiple Comparisons—Liver Weight and Fat Visibility

Significant difference in liver weights was shown between diet groups (p <0.001) and between diet*treatment groups (p = 0.026). This led to a LSD posthoc test showing specific differences between diet groups and diet*treatment groups regarding liver weight.

Significant difference in liver weight was shown between the 4% and 11% diet groups dosed with water (p <0.001) and between the 4% and 11% diet groups dosed with acetaminophen (p <0.001). Significant difference in liver weight was also shown between the 11% diet group dosed with water and the 11% diet group dosed with acetaminophen (p <0.001). This indicates a difference in liver weight due to diet as well as diet*treatment with acetaminophen.

Significant difference in visible fat content was shown between diet groups (p <0.001) and between treatment groups (p = 0.001). This led to a LSD posthoc test showing specific differences between diet groups and treatment groups regarding visible fat content. Significant difference in visible fat content was shown between the 4% and 11% diet groups dosed with water (p = 0.001) and between the 4% and 11% diet groups dosed with acetaminophen (p <0.001).

Significant difference in visible fat content was shown between the 4% group dosed with water and the 4% group dosed with acetaminophen (p <0.001). Significant difference was also seen between the 11% group dosed with water and

the 11% group dosed with acetaminophen (p <0.001). This indicates a difference in visible fat content due to diet as well as treatment with water or acetaminophen.

Chapter V

DISCUSSION

The primary purpose of this study was to determine the effects of acetaminophen-induced liver toxicity and obesity-induced liver toxicity. The hypothesis of this study was: the effects of acetaminophen dosages in the high fat-fed mice will show greater indications of liver damage than mice on a normal fat-fed diet. This is because (1) the liver will see a higher dose in the obese mice which will cause liver damage and (2) the liver cells will become fatty from the high-fat diet which will also harm the liver. A secondary purpose of this study was to determine the relationship between diet-induced liver damage and drug-induced liver damage.

The hypothesis was partially accepted due to the significant damage seen on the high-fat fed mice livers from the GDH activity assay. Damage to liver mitochondria releases GDH, so that a higher GDH activity level signifies higher levels of liver damage. Higher levels of GDH activity were seen in the high-fat diet mice groups.

However, in this study there was no significant difference in GDH activity levels between treatment groups (water vs. APAP) and diet*treatment groups. This indicates damage was done to the liver due to high fat diet alone.

Other studies have shown damage to the liver by measuring GDH levels in the overdose of acetaminophen. McGill and colleagues researched the mechanisms of acetaminophen toxicity in humans. Their group found biomarker levels of

mitochondrial damage (GDH levels and mitochondrial DNA) and nuclear DNA fragments were increased in plasma levels from APAP-overdose human patients (2012). McGill and colleagues tested blood samples from 40 patients, 20 with ALT levels of 1,000 U/l or more (signifying increased APAP toxicity) and 20 with ALT levels of less than 1,000 U/l (signifying less or none APAP toxicity). They used blood samples from 6 healthy volunteers as controls. The mean age of their study was 38 years old with the majority of their patients being female. Overall, the average GDH activity (signifying its release after mitochondrial membrane lysis from necrotic cells) was increased in the APAP-overdose patients.

Figure 8 is a scatter plot representation of GDH activity levels plotted versus liver weights. This shows the general relationship of an increased liver weight corresponding to an increased GDH activity level. While the GDH activity level is related primarily to mitochondrial dysfunction, it is interesting to note the relationship between the two variables.

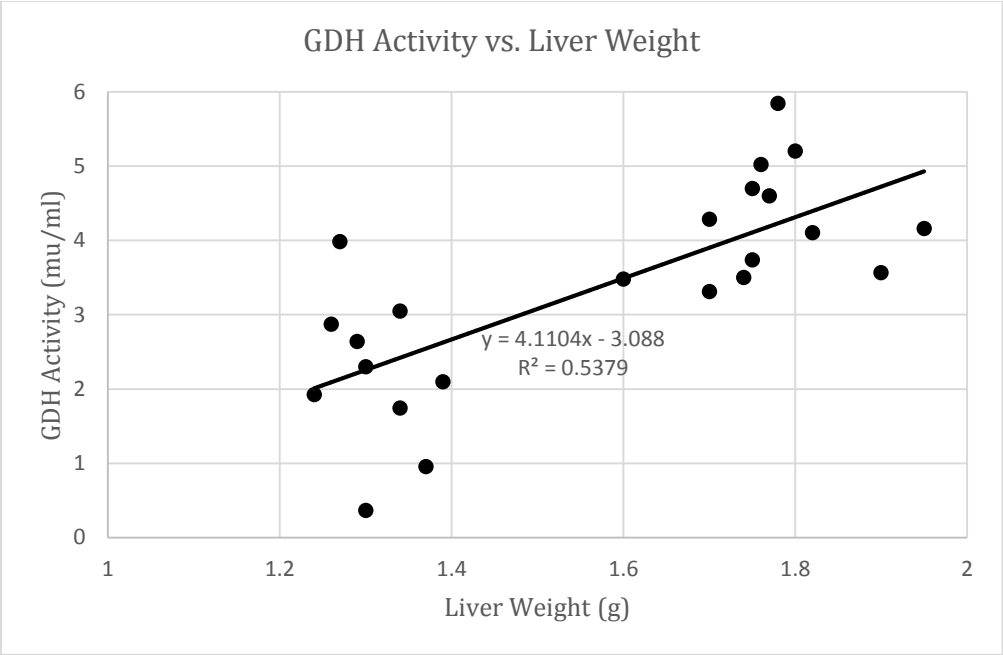


Figure 8. *GDH Activity vs. Liver Weight.*

McGill and colleagues later conducted a similar study (2014). In this second study, their group looked at the same biomarker levels (GDH, mtDNA, and nDNA fragments) in serum from non-survivors of APAP-induced acute liver failure, compared to survivors. They found that all three biomarker levels were significantly increased in patients who died, compared to those who survived (GDH: 450 ± 73 vs. 930 ± 145 U/L; mtDNA: 21 ± 6 vs. 48 ± 13 and 33 ± 10 vs. 43 ± 7 ng/mL for two different genes; nDNA fragments: 148 ± 13 vs. 210 ± 13% of control). It is important to note that McGill conducted these studies on humans, whereas previous studies had only been conducted in rodent models. Their group found similar APAP-overdose effects in humans that previous studies have shown in rodent models.

The work of McGill and colleagues demonstrated that the mitochondria are important players in the mechanisms of APAP hepatotoxicity in humans. Serum GDH, mtDNA, and nDNA fragments will provide useful information in predicting patient outcome after APAP overdose (2014).

The most surprising result of the present study was the increased GDH activity seen in the high-fat fed diet control group dosed with water. This implies that significant damage was done to the liver due to high-fat diet, or obesity, alone. Other research has looked at the effects of high-fat diet, non-alcoholic fatty liver disease and acetaminophen overdose.

Nguyen and colleagues researched APAP overdose in patients with non-alcoholic fatty liver disease, NAFLD (2008). In their study, patients with NAFLD who were hospitalized with APAP overdose had more than a seven-fold higher prevalence of liver injury when compared to those patients without NAFLD. In summary, Nguyen and colleagues found that severe acute liver injury after APAP overdose was increased with NAFLD and alcoholic liver disease.

Another study done by Myers and Shaheen (2009) analyzed the same database surrounding APAP overdose in patients with NAFLD, as well as taking into account differences in overdose circumstances. Their study supported the work done by Nguyen (2008) in finding increased levels of liver damage from NALFD patients with APAP overdose.

Acetaminophen overdose is associated with not just liver damage, but also damage to other organs, including the kidneys. Corcoran and Wong (1987) showed

how obesity was an extreme risk factor in increasing liver and kidney damage in the obese fed rat. They showed how increased obesity was directly associated with increased toxicity in not only the liver, but also the kidney as well. Further interest may to investigate acetaminophen's metabolism in the hepatic-renal function of obese individuals.

Both studies by Nguyen (2008) and Myers (2009) implied increased liver damage due to NAFLD in acetaminophen overdose patients. However, it is still unclear whether obesity enhances the severity of liver damage alone. Mechanisms involving the extent of liver damage due to obesity should be further studied to investigate liver damage of APAP-overdose patients due to obesity alone. The present study would suggest that obesity alone does in fact harm the liver just as much as damage from by acetaminophen overdose.

The table on the following page summarizes several studies identifying APAP-induced acute liver injury in different animal models taking into account normal diet, high-fat diet, and the presence of NAFLD (Michaut, 2014). Four of the studies showed that the presence of NAFLD was related with higher liver injury after a single APAP overdose (Kon, et al., 2010; Kucera et al., 2012; Aubert et al., 2012; and Donthamsetty, 2008).

These studies showed the presence of NAFLD in obese individuals increased the risk of APAP-induced liver injury. Two studies showed similar or lower APAP hepatotoxicity in some rodent models of obesity (Blouin, et al., 1987; Ito, et al., 2006).

It seems that APAP-induced liver injury in an obese individual depends on a balance between several metabolic factors. These factors can be protective such as higher APAP glucuronidation and volume of distribution, and lower absorption rate of NAPQ1. Other metabolic factors can be destructive such as increasing hepatic production of NAPQ1, or the decrease in detoxification of NAPQ1 by lowering GSH stores (Michaut, 2014).

Further studies must look at the mechanisms behind obesity-induced liver toxicity as the present study showed there is significant damage done to the liver by high-fat diet alone.

References	Animal Models of NAFLD	Presence of NAFLD	Dose of APAP	Response to APAP toxicity
Corcoran and Wong, 1987	Male rats fed with a high-fat diet for 24 weeks	Not reported in this study	710 mg/kg (i.p.)	Higher toxicity after 48 h, compared to rats fed standard diet
Blouin, et al., 1987	Obese male rats	Not reported in this study	1300 mg (p.o.)	Similar toxicity after 48 h, compared to lean rats
Ito, et al., 2006	Male rats fed a normal and high-fat diet	Yes	300 mg/kg (p.o.)	Lower toxicity after 6 h, compared to mice fed a standard diet
Kon, et al., 2010	Male mice	Yes	300-600 mg/kg (p.o.)	Higher toxicity after 6 h, compared to wild-type mice
Kucera, et al., 2012	Male mice rats fed a high-fat diet for 6 weeks	Yes	1 g/kg (p.o.)	Higher toxicity after 24 and 48 h, compared to rats fed a standard diet
Aubert, et al., 2012	Female diabetic and female obese mice	Yes	500 mg/kg (o.p.)	Higher toxicity after 8h, compared to wild-type mice
Donthamsetty, 2008	Male mice fed a high-fat diet	Yes	360 mg/kg (i.p.)	Higher toxicity from 6 to 48 h after overdose, compared to mice fed a standard diet

Table 6. *Studies of APAP-induced liver injury and NAFLD (Michaut, 2014).*

Studies have shown that weight and fatty liver content correlate to increased levels of disease in obese individuals (Horvath et al., 2014; Faghihzadeh et al., 2014). Faghihzadeh and colleagues showed that administering resveratrol decreased liver weight, serum liver enzymes, inflammatory markers, hepatic steatosis and fibrosis. The administration of resveratrol, as well as modifying lifestyle and eating habits, was shown to decrease signs of liver damage including fatty liver (2014). Our present study suggests that increased liver weight and signs of fat in the liver would correspond to a higher level of liver disease.

Horvath and colleagues studied how the liver ages in response to obesity. Their group found specific signs of aging, including liver weight and fat visibility, to increase the age of the liver leading to liver disease, such as NAFLD, earlier in life. Their research suggests that liver aging due to obesity alone is accelerated due to processes occurring in the liver involving oxidative stress and energy metabolism (2014).

Our present study would confirm that obesity does in fact age the liver by showing signs of fatty liver (increased weight and visible fat) and leading to further disease. Horvath and colleagues would propose that oxidative stress and changes in energy metabolism of liver cells would increase the fat content of the liver (2014).

Acetaminophen overdose is a dangerous problem in developing males and females. Most studies use male rodent models, where female models may be overlooked as an important model to use to view how hepatotoxicity and fat toxicity affects development.

The present study showed that high-fat diet alone damages liver mitochondria shown through the increased levels of GDH in high-fat diet groups. This may be especially true for female developing bodies. However, the present study did not show difference in GDH levels, and mitochondria damage, between acetaminophen-dosed and water-dosed mice groups, as previous research has shown. Several factors could have been the cause of this discrepancy and would require further study:

1. The mice were dosed orally (p.o.) and the exact dosage of acetaminophen may have been altered due to dosing techniques.
2. The mice used in the present study were female. Previous research has shown that male mice show greater sensitivity to acetaminophen-induced liver injury than females (Mohar et al., 2014).

The first experiment of measuring GHD levels reflected the effects of high-fat diet on the mice, and the damage done to liver mitochondria due to fat alone. A high-fat diet most likely damaged female mice liver mitochondria due to lipid accumulation and possible inflammatory effects. This study showed that acetaminophen was less toxic in female mice and was not sufficient in these experiments to show leakage of GDH.

The second measurement of liver weights indicated that acetaminophen exacerbated liver hypertrophy in the high fat group only. The damage done to cytoplasmic structures such as the endoplasmic reticulum, etc. may be responsible for this effect which is not reflected in the leakage of GDH by the mitochondria.

The third measurement of fat accumulation in livers caused by acetaminophen is likely a damage-induced steatosis and was clearly evident in the high-fat diet groups. This confirms and augments the liver weight analysis.

In summary, these different measured parameters (GHD, liver weight, visible fat) signaled different damaging effects in the liver. They all indicated obesity is very problematic for liver function (significant diet category). The differences in liver weight indicated additionally that obesity may increase acetaminophen toxicity (diet*treatment interaction category). The liver fat visibility test indicated that fat accumulation in the liver increased by abusing acetaminophen (treatment alone category).

Fat toxicity may be extremely harmful on adolescent female bodies. This study proved the damaging effects done to the liver due to fat toxicity alone. Previous research suggests that administering acetaminophen incorrectly (based on weight or other reasons) would also be toxic to the liver.

Further research may look into the toxicological effects of fat on the female liver, as well as other organ systems. Since females have a higher percentage body fat than males, implications to the female reproductive system may also be a point of examination for future research. In particular, if an individual is overweight and female, the severity of fat toxicity throughout the body may be largely understudied and overlooked.

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