AMERICAN UNIVERSITY OF BEIRUT

# THE EFFECT OF PHOSPHORUS MANIPULATION ON THE PROGRESSION OF NON-ALCOHOLIC FATTY LIVER DISEASE IN RATS MAINTAINED ON A HIGH FRUCTOSE DIET

by
JINANE MOHAMAD MAKKI

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# AN ABSTRACT OF THE THESIS OF

Jinane Mohamad Makki for: Master of Science
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Title: The effect of phosphorus manipulation on the progression of non-alcoholic fatty liver disease in rats maintained on a high fructose diet

Non Alcoholic Fatty Liver Disease (NAFLD) is an emerging disease associated with metabolic dysfunction. It is characterized by the excessive accumulation of lipids in the form of triglycerides in hepatic tissues of patients who do not consume large amounts of alcohol.

High fructose feeding has been used to develop NAFLD, which is reported to be associated with Fructose intake. Fructose is known to sequester phosphorus and making it unavailable for the production of ATP. At this point, attention is raised to the role of phosphorus in fructose-induced fatty liver disease and to what extent the manipulation of phosphorus can affect this disease.

The aim of the following study is to investigate the effect of different phosphorus-content diets on rats having high fructose intake, to test for the role of phosphorus in the development of NAFLD

Following adaptation, forty three male Sprague-Dawley rats were randomly divided into one control group (30% sucrose and 30%starch) and four high fructose (60%) groups with different levels of phosphorus: 0.15%-0.3%-0.6% and 0.9%. After 10 weeks rats were sacrificed by decapitation. Food intake and body weight was measured twice per week. Liver biopsy along with plasma analysis was performed. Steatosis was assessed by histologic examination of Hematoxylin and Eosin (H&E) stained liver tissue sections, whereby macrovesicluar steatosis was graded from 0 to 3 and microvesicular steatosis from 0 to 1. Histologic quantitation of steatosis was also performed by image analysis of liver sections stained with Oil Red O.

The results showed that high fructose diet reduced body weight by 9% compared to control group. High fructose diet did result in any macrovesicular steatosis. Microvesicular steatosis was more frequent in the high phosphorus groups. A Zone 3 distribution was the most common pattern in HF/0.15%P and HF/0.3%P groups while zone 1 distribution of steatosis dominated the HF/0.6%P and HF/0.9%P groups.

In this study, maintaining rats on high fructose diet did not induce significant steatosis. Therefore, the impact of phosphorus on the progression of NAFLD is not demonstrated.

Keywords: NAFLD, Fructose, Phosphorus, ATP, Rats

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# ABREVIATIONS

| | |
|---|---|
| ALT | Alanine transaminase |
| AMP | Adenosine Monophosphate |
| ApoB | Apolipoprotein B |
| AST | Aspartate Aminotransferase |
| ATP | Adenosine Triphosphate |
| BMI | Body Mass Index |
| ChREBP | Carbohydrate-responsive element-binding protein |
| $CO_2$ | Carbon dioxide |
| EDTA | Ethylene diamine tetra-acetic acid |
| EPD | Epididymal fat pad |
| ELISA | The enzyme-linked immunosorbent assay |
| ER | Endoplasmic reticulum |
| GLUT2 | Glucose transporter 2 |
| GLUT5 | Glucose transporter 5 |
| HF | High fructose |
| HP | High phosphorus |

| | |
|---|---|
| LPL | Lipoprotein lipase |
| MCD | Methionine-choline deficient |
| NAFL | Non-Alcoholic Fatty Liver |
| NAFLD | Non-Alcoholic Fatty Liver Disease |
| NASH | Nonalcoholic steatohepatitis |
| NHANES | The National Health and Nutrition Examination Survey |
| NO | Nitric oxide |
| OTC | Over-the-counter |
| P | Phosphorus |
| PDH | Pyruvate Dehydrogenase |
| PFK | Phosphofructokinase |
| Pi | Inorganic phosphate |
| PTH | Parathyroid Hormone |
| ROS | Reactive oxygen species |
| TCA | Tricarboxylic acid cycle |
| TG | Triglyceride |
| TLR4 | Toll-like receptor 4 |
| TNFα | Tumor necrosis factor alpha |

| | |
|---|---|
| UCP2 | Uncoupling protein 2 |
| VLDL | Very low density lipoprotein |

# CHAPTER I

# INTRODUCTION

Non Alcoholic Fatty Liver Disease (NAFLD) is an emerging disease associated with metabolic dysfunction. It is characterized by the excessive accumulation of lipids in the form of triglycerides in hepatic tissues of patients who consume little or no alcohol. NAFLD ranges from simple steatosis, to steatosis accompanied by inflammation and fibrosis (steatohepatitis or NASH) that can further develop into cirrhosis or hepatocellular carcinoma (HCC). (Yilmaz, 2012)

The pathogenesis of NAFLD has not been entirely revealed; therefore knowledge of intracellular mechanisms that trigger NAFLD is of greatest importance. NAFLD can take decades to evolve and progress which limit the quality of clinical studies. Consequently, researchers have turned their attention to the development of suitable animal models to test the role of a gene or dietary factor in fatty liver formation. Animals that are fructose loaded for several weeks are widely recognized as good models for NAFLD.

Absorbed fructose reaches quickly the liver and unlike glucose binds irreversibly to the phosphate molecule to produce fructose-1- phosphate. By this process, fructose will be bypassing the enzyme phosphofructokinase (PFK) which is the key regulatory enzyme for glycolysis. Thus the system will proceed toward lipogenesis (TG formation), since there is no negative feedback mechanism regulating the phosphorylation of fructose. (Tappy et al, 2010)

Sometimes , it could be that when large doses of fructose are ingested, inorganic phosphorus coming from ATP is taken to produce fructose-1-phosphate resulting in low hepatic ATP levels, therefore intracellular phosphate levels fall resulting in the stimulation of AMP deaminase which drive AMP toward the production of uric acid. (Nakagawa et al, 2006)

Cellular ATP depletion can cause an arrest in protein synthesis and induce inflammatory and pro-oxidative changes. Also, it is suggested that uric acid may itself have pro-inflammatory and oxidative effects. ATP depletion may also explain the proposed associations of increased fructose consumption with increased hepatic inflammation.

Until now no previous study has examined the role of phosphorus in hepatic ATP depletion, which consequently results in fatty liver. At this point, attention is raised on the role of phosphorus in fructose induced fatty liver disease and to the extent the manipulation of phosphorus can affect this disease. Is fructose-induced fatty liver disease dependent on phosphorus?

Therefore, manipulating the phosphorus content of the diet could help to test whether phosphorus is a factor in the development of NAFLD.

# CHAPTER II

# LITTERATURE REVIEW

## A. Fructose : Overview

Fructose is a simple monosaccharide with the same molecular formula as glucose but with a different structure. They are classified differently as hydrocarbon derivatives: glucose being classified as an aldehyde and fructose as a ketone. (Gibson et al, 2007) There are three main sources of fructose in our diet: it can be found as free fructose in fruit and honey or bound to glucose forming sucrose or high fructose corn syrup (HFCS).(Akram et al,2013) In recent decades, the intake of HFCS has increased dramatically. HFCS is used widely in carbonated beverages, canned fruits, sweetened drinks, baked goods, candies, jams, jellies and dairy products. It consists approximately of 55% fructose and 45% glucose. (Tappy et al, 2010) Food manufacturers often prefer HFCS to sucrose because it is inexpensive, presents good stability in acidified foods and drinks, and its storage and transport is easier than sucrose and it mixes well in many foods.

Unlike sucrose and glucose, fructose is considered a safe form of sugar since it does not require insulin for uptake into cells and moderate fructose intake does not adversely affect glycemic control. However, a concern has been raised because several pieces of evidence suggested that excessive fructose intake is responsible in part for the

increasing prevalence of obesity, type 2 diabetes mellitus and non-alcoholic fatty liver diseases.(Bray et al, 2004)

## B. Fructose metabolism :

### *1. Fructose metabolism in the Gut*

Fructose is transported into the enterocyte through a specific fructose transporter, GLUT5, located at the apical membrane of the enterocyte. Unlike glucose, this process does not require ATP hydrolysis and is independent of sodium absorption. The entry of fructose into the bloodstream occurs through GLUT2 at the basolateral membrane of the enterocyte. (Tappy et al, 2010) Fructose absorption appears to be quantitatively limited. Therefore, the consumption of high amount of fructose can exceed the capacity of intestinal fructose absorption, resulting in diarrhea and flatulence. (Rumessen et al, 1986)

Once inside the enterocyte, fructose undergoes different possible conversions:

- Part of fructose appears to be converted into lactate and released into the portal circulation.
- Fructose is converted into glucose by the enzyme glucose-6-phosphatase (intestinal gluconeogenesis)

- Some species can convert fructose into fatty acids within the enterocyte (intestinal de novo lipogenesis). Whether a similar pathway is active in humans remains however unknown. (Tappy et al, 2010)

### *2. Hepatic Metabolism*

Absorbed fructose is transported via the portal vein to the liver. Fructose uptake in the liver is thought to be operated by the glucose transporter GLUT2. At this site, fructose is metabolized by fructokinase to fructose-1-phosphate. This molecule is cleaved by aldolase B to form the triose phosphates: dihydroxyacetone phosphate and glyceraldehydes. (Figure 1) Unlike glucose metabolism, fructose conversion to triose-P occurs independently of insulin and is a rapid process due to the low Km of fructokinase for fructose, and to the absence of negative feedback by ATP or citrate. This leads to a transient depletion of free phosphate and a decrease in ATP in liver cells in response to fructose.

Triose-P produced from fructose can be converted into:

- Pyruvate and oxidized into $CO_2$ and $H_2O$ in the tricarboxylic acid cycle.
- Lactate and released into the systemic circulation
- Glucose and glycogen through gluconeogenesis (major portion)
- Fatty acids in hepatocytes through the process of de novo lipogenesis

Another metabolic effect of fructose is exerted through an increased intrahepatic fructose-1-P concentration. This rise in fructose-1-P has important indirect effects on

hepatic glucose metabolism by modulating glucokinase activity. Fructose-1-P, at low concentration, enhances glucokinase activity. (Tappy et al, 2010) Hepatic glucokinase is a key regulatory enzyme in hepatic glucose metabolism, since it is required for the formation of glucose-6-P. Thus Glucokinase is responsible for glucose uptake by the liver. As a consequence, addition of small, so-called "catalytic" doses of fructose to a glucose meal can enhance hepatic glucose disposal. (Donmoyer et al, 2001)

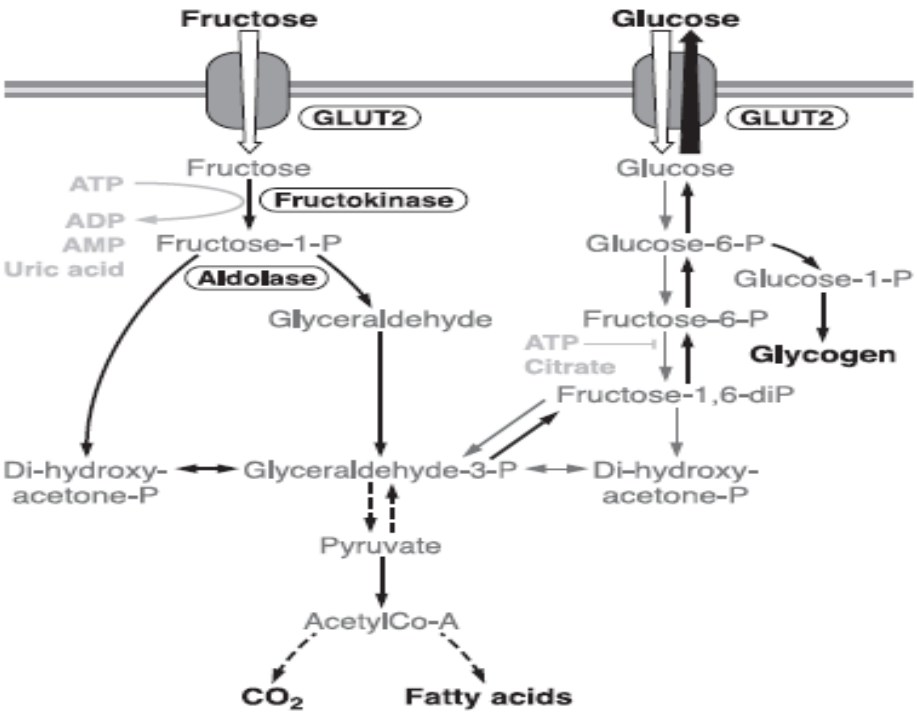



**Figure 1:**Fructose metabolism in liver cells.(Tappy et al, 2010)

## C. Absorption capacity of fructose in healthy adults

Rumessen and Hoyer (1986) found that in the healthy state, large individual variations exist in the absorption capacity of fructose, and that the total absorption capacity is even lower than previously believed. Some healthy individuals may fail to absorb as little as a 5 g dose. Furthermore, they showed that ingestion of fructose as sucrose does not result in significant malabsorption and that the addition of glucose to fructose solutions promotes fructose absorption in a dose dependent manner. Fructose malabsorption may be considered a normal phenomenon in the healthy state and up to 30-40 g fructose may be malabsorbed without causing significant abdominal discomfort. (Rumessen et al, 1986) When the fructose dose was increased from 25 to 50g (10% solution) the prevelance of incomplete absorption increased from 0 to 37.5%, 11 to 58%, and 50 to 80 % respectively in three different studies. These data suggest that for a 10% solution, the threshold for fructose absorption in most healthy individuals lies between 25 and 50 g. (Skoog et al , 2004)

## D. Long term effect of fructose:

### *1. Dyslipidemia:*

Fructose is highly lipogenic since it provides large amounts of hepatic triose-phosphate as precursors for fatty acid synthesis. Several studies showed that fructose

consumption increases hepatic de novo lipogenesis, which in turn upregulates VLDL production and secretion. In addition, fructose contributes to the synthesis of both the glycerol- and the fatty-acyl parts of VLDL-triglycerides. (Figure:2) As well fructose may increase the expression of key lipogenic enzymes in the liver.

Fructose induces the expression of the factor of transcription SREBP-1C , the principal inducer of hepatic lipogenesis, independently of insulin. Also, it activates the hepatic transcription factors carbohydrate-responsive element binding protein (ChREBP), which upregulates the expression of hepatic fatty acid synthase and acetyl-CoA carboxylase. (Lustig , 2010)

Another mechanism involved in fructose induced dyslipidemia is the impaired clearance of triglyceride-rich lipoprotein. ApoCIII is an inhibitor of LPL and hepatic lipase and plays a pivotal role in the hydrolysis and clearance of TG rich particles such as VLDL and chylomicron. In vivo studies demonstrated that high fructose intake leads to an increase in ApoCIII production which causes impaired TG hydrolysis. (Dekker et al, 2010) Bantle et al (2000) showed that a fructose diet produced significantly higher plasma triglycerides than did a glucose diet in men on days 7, 28 and 42. In addition, daylong plasma triglycerides on day 42 were 32% higher in men during the fructose diet than during the glucose diet.( Bantle et al, 2000)

Both animal and human studies point out a gender difference in fructose-induced hypertriglyceridemia: chronic high-fructose diets caused hypertriglyceridemia in male rats, while female rats appeared protected against fructose induced changes in metabolism. This

protection was no longer present after oophorectomy, suggesting that female sex hormones may confer protection against the effects of a fructose diet. (Tappy et al, 2010)

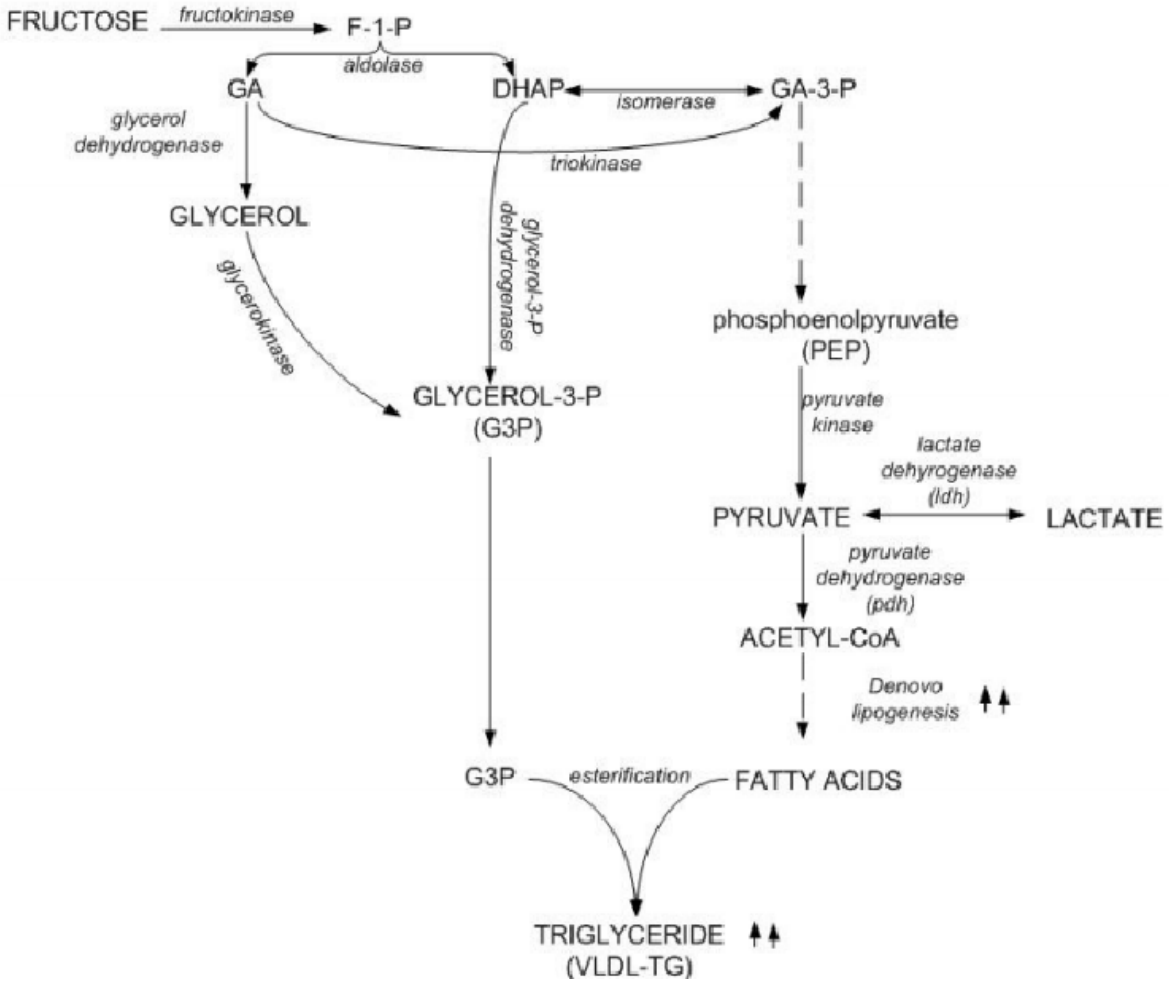



**Figure 2 :** Fructose metabolism: A highly lipogenic pathway

### *2. Ectopic lipid deposition in the liver and skeletal Muscle*

Ectopic lipid deposition is the deposition of TG in the cytoplasm of non-adipose cells, such as hepatocytes, muscle fibers, or endocrine cells. In addition to dyslipidemia, fructose may modulate intracellular lipid deposition (Le et al, 2009).

At the molecular level it was suggested that the mechanisms behind this effect of fructose are: inhibition of PPARα in liver cells and stimulation of hepatic de novo lipogenesis through enhanced intrahepatic synthesis of triose phosphate precursors and increased expression of lipogenic genes and reduced hepatic lipid oxidation.( Roglans et al , 2007)

Pagliassotti et al (1996) showed that in rodents, a high-sucrose diet rapidly, within 1 week, increased intrahepatic fat deposition.

In addition to dyslipidemia, the study of Le et al (2009) showed that fructose increased intrahepatocellular lipid (IHCl) and intramyocellular lipid (IMCL) concentration by 76% in healthy individuals. (Le et al, 2009). The increase in IHCls is best explained by the stimulation of de novo lipogenesis by the high fructose diet. (Le et al, 2009).

### *3. Hyperuricemia:*

Nakagawa et al (2006) showed that serum uric acid levels were elevated in fructose fed rats compared with rats fed a normal diet at 4 week. The rapid phosphorylation of fructose use ATP as a phosphorus provider leading to reduced cellular ATP. Therefore intracellular phosphate levels fell, resulting in the stimulation of adenosine monophosphate (AMP) deaminase. Consequently the increase stimulation of AMP deaminase pushes AMP

toward the production of uric acid as opposed to the regeneration of ATP by AMP kinase. (Nakagawa et al, 2006)

In addition to an effect of fructose in increasing hepatic production of uric acid, it was found that urinary excretion of uric acid was decreased in fructose fed rats. (Nakagawa et al, 2006) This observation could be due to the production of lactate from fructose metabolism which is a competitive inhibitor for urate excretion. (Nakagawa et al, 2006) The third NHANES report indeed indicates that consumption of sugar-sweetened beverages is significantly associated with plasma uric acid concentrations.(Tappy et al,2010)

***4. Insulin resistance:***

Several studies have pointed to the harmful effect of fructose on glucose metabolism and insulin sensitivity. Gerald Reaven showed that substituting fructose for the carbohydrate present in rat chow led to insulin resistance, hyperinsulinemia and hypertriglycemia in Spargue-Dawley rats. These changes were seen within 1 week and were not associated with obesity. (Shulman 2000) Insulin resistance is strongly linked to lipid metabolism disorders. Ectopic lipid deposition may generate toxic lipid-derived metabolites, such as diacylglycerol, fatty acyl CoA, and ceramides. The presence of these metabolites in the intracellular environment leads to a higher serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1), which has been shown to reduce insulin signaling. (Shulman, 2000)

It was further observed that fructose induced insulin resistance through the activation of the C-Jun N terminal kinase (JNK). (Tappy et al, 2010)

Fructose could also decrease insulin sensitivity through changes in the gut microbial flora and alterations of intestinal permeability. Fructose results in an increase of plasma concentration of bacterial lipopolysaccharides, or endotoxin. Low-grade endotoxinemia in turn activates inflammatory pathways and impairs insulin's action, leading to the development of insulin resistance. (Tappy et al, 2010)

Moreover, fructose may cause insulin resistance through increased uric acid production which contributes to so-called prereceptor insulin resistance. Insulin enhanced glucose utilization through the stimulation of key metabolic pathways in insulin-sensitive cells, and through an increase in blood flow and nutritive circulation to the major insulin-sensitive tissue, skeletal muscle. This effect of insulin is due to the activation of the endothelial enzyme nitric oxide synthase (eNOS). (Tappy et al, 2010) Since eNOS is inhibited by uric acid, it was proposed that inhibition of the vascular effects of insulin by uric acid was involved in fructose-induced insulin resistance. In support of this hypothesis it was reported that, in rats fed a high-fructose diet, both hyperuricemia and insulin resistance develop simultaneously. Furthermore, the development of insulin resistance was prevented by lowering uric acid concentrations with a uricosuric agent. (Nakagawa et al, 2006). (Figure: 3)

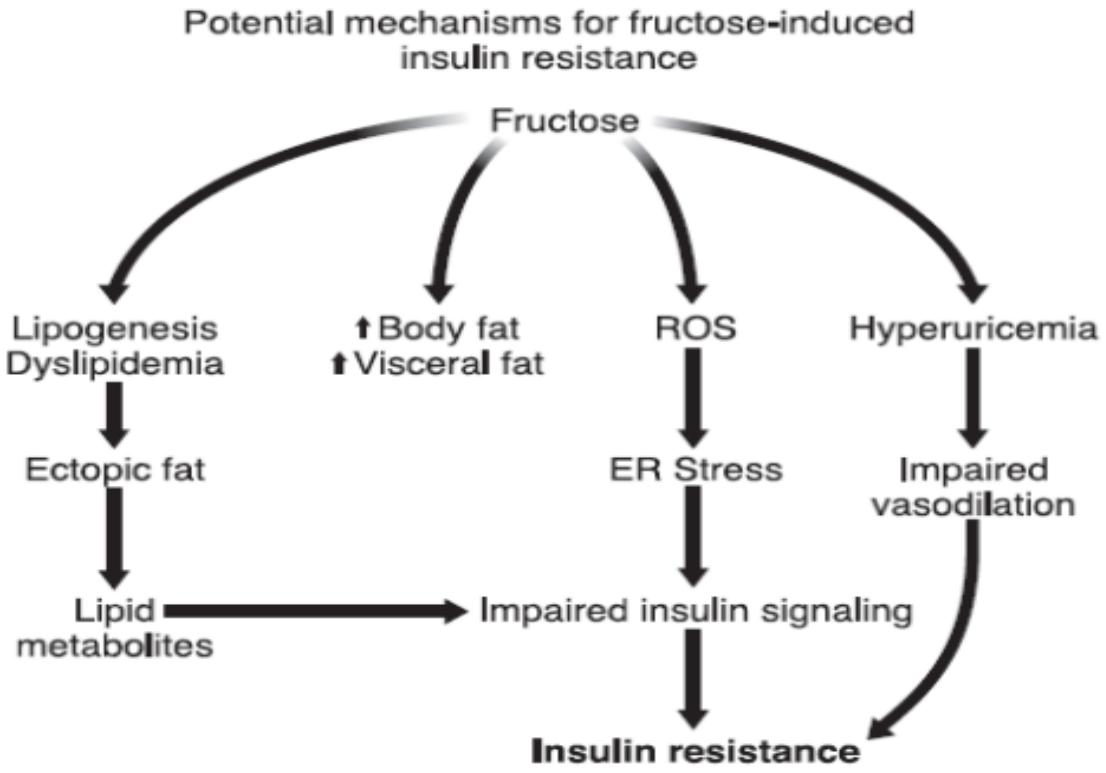



**Figure 3:** Summary of the potential mechanism for fructose-induced insulin resistance (Tappy et al, 2010)

## E. Phosphorus: Background and Function:

Phosphorus is mainly found in nature in its pentavalent form in combination with oxygen, as phosphate ($PO_4^{3-}$).

Phosphorus makes up about 0.5 percent of the newborn infant body (Fomon and Nelson, 1993), and from 0.65 to 1.1 percent of the adult body (Aloia et al., 1984; Diem, 1970). Eighty-five percent of total body phosphorus is present in bones. The remaining 15 percent is distributed in soft tissues (14%) and extracellular fluid (1%). (Gattineni et al, 2012) Phosphorus concentration in the extracellular space is determined by the interactions among intestinal absorption, renal excretion, and exchanges with bone and the intracellular space. (Uribarri, 2007) (Figure: 4)

Phosphorus plays both structural and functional roles in human body. Structurally, phosphorus occurs as phospholipids nucleotides and nucleic acids.

The functional roles include:

- Helps body to maintain normal PH through the buffering of acid or alkali excess
- ATP synthesis, which is the source of energy for many cellular reactions
- Red cell 2,3-diphosphoglycerate (2,3-DPG) synthesis, which regulates the dissociation of oxygen from hemoglobin
- The activation of many catalytic proteins by phosphorylation (Bilezikian et al, 2002)

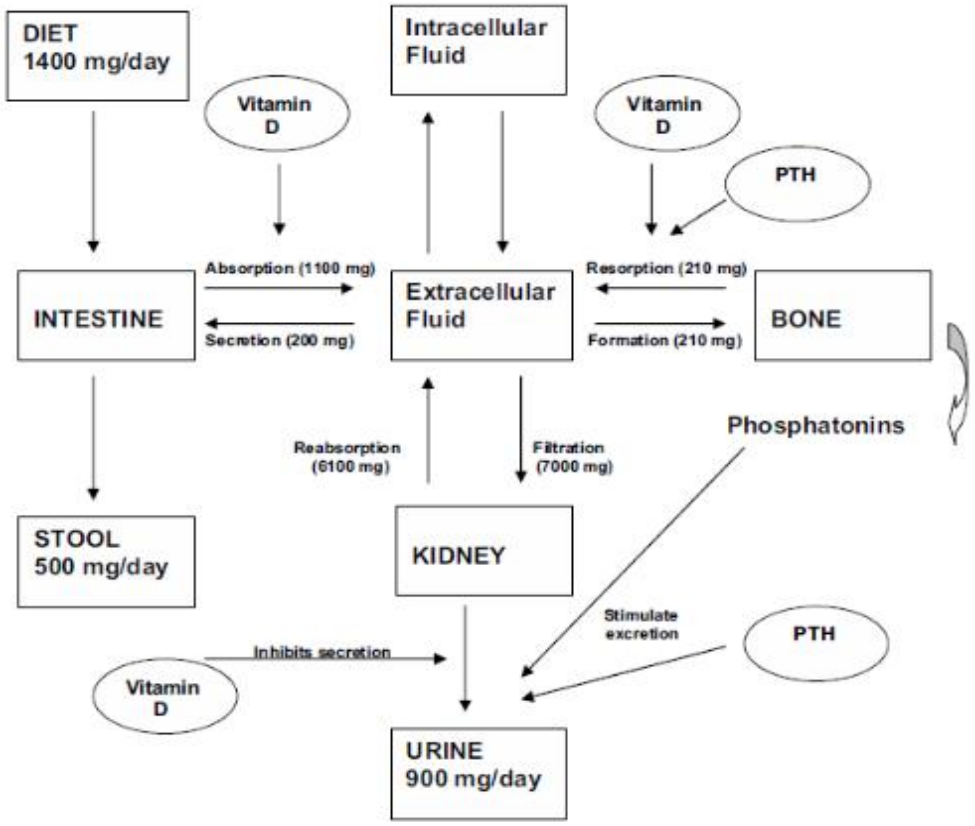



**Figure 4:** Summary of phosphate metabolism for a normal adult in neutral phosphate balance ( Gaasbeek and Meinders,2005)

## F. Sources of Dietary phosphorus:

The most important determinant of serum phosphorus is the amount of phosphorus absorbed. Phosphorus absorption is governed by the total amount of phosphorus in the diet and its bioavailability. Total dietary phosphorus intake is derived from three different sources:

1- Natural sources of phosphorus: unlike dietary calcium, phosphorus is found in most foods especially in high protein foods like: milk, meat, poultry and fish, grain products and legumes. These foods are often referred to as organic phosphorus. (Calvo et al, 2013)

NHANES showed a consistent relationship between high protein foods and natural phosphorus content, and showed a mean total intake of 15-17 mg of phosphorus for every gram of protein.

2- Inorganic phosphorus in OTC medications, dietary supplements, food enrichment and water. These sources are an unseen source of dietary phosphorus. (Calvo et al, 2013)
3- Food additives and ingredients used in processing: in the US and other western cultures, phosphorus that is added during processing represent an average of 500mg/day per capita daily intake. Most phosphorus containing additives are inorganic salts of phosphorus.

## G. Phosphate homeostasis

### *1. Absorption:*

Food phosphorus is a mixture of inorganic and organic forms. Most phosphorus absorption occurs as inorganic phosphate. The absorption of the organic forms is facilitated by the intestinal phosphatases which hydrolyze the organic form of phospahate to the inorganic form which is the form absorbed. Absorption occurs throughout the small

intestine with transport greatest in the jejunum and ileum and less in the duodenum. Essentially no absorption occurs in the colon. (Bilezikian et al, 2002) The absorption of phosphorus in the intestine is dependent on the amount and availability of phosphorus present in the diet. In normal subjects, net P absorption is a linear function of dietary P intake. Indeed, for a dietary P range of 4 to 30 mg/kg/day, the net P absorption averages 60 to 65% of the intake. (Lee et al , 1986)

A portion of phosphorus absorption occurs through a saturable , active transport facilitated by 1,25-dihydroxyvitamin D (1,25(OH)2D). However, the bulk of phosphorus absorption occurs by passive, concentration-dependent processes. (Gattineni et al , 2012) Phosphorus absorption is reduced by ingestion of aluminum-containing antacids and by pharmacologic doses of calcium carbonate. However at normal intakes, there is no significant interference of calcium with phosphorus absorption. .( DRI, 1997)

***2. Excretion:***

The main route of phosphorus excretion is through the kidneys. Inorganic serum phosphate is filtered at the glomerulus and reabsorbed in the proximal tubule. Phosphorus excretion is regulated by PTH .The transport capacity of the proximal tubule for phosphorus is limited. This limit is called the tubular maximum for phosphate (TmP). TmP varies inversely with parathyroid hormone (PTH) concentration. When the filtered load is less than the TmP (low plasma P*i* values), most or the entire filtered load is reabsorbed, and thus plasma phosphate levels can be maintained. ( DRI, 1997)

In healthy adults, urine phosphorus is equal to absorbed diet phosphorus, less small amounts of phosphorus lost in shed cells of skin and intestinal mucosa. ( DRI, 1997) The major factors known to increase renal tubular phosphorus reabsorption include phosphate depletion, 1, 25$(OH)_2$ vitamin D, volume depletion, metabolic alkalosis, chronic hypocalcemia and hormones such as insulin, estrogen, thyroid hormone, and growth hormone. (Uribarri, 2007) However, PTH, phosphatonins, acidosis, hyperphosphatemia, chronic hypercalcemia and volume expansion lead to a decrease in phosphorus tubular reabsorption. (Uribarri, 2007)

## H. Non-alcoholic Fatty liver disease

### *1. Definition*

Non-alcoholic fatty liver disease (NAFLD) is a common but often silent chronic liver disease characterized by the excessive accumulation of lipid in the form of triglycerides in hepatocytes occurring in people who consume little or no alcohol.( Yilmaz, 2012) NAFLD used to be exclusively a disease of adults but now is becoming a major health concern also in obese children. The prevalence of childhood obesity has significantly increased over the past three decades and boosted the prevalence of NAFLD in adolescents. (Fon Tacer et al, 2011)

NAFLD comprises a wide spectrum of histological lesions ranging from simple steatosis to co-existent inflammation with hepatocyte ballooning and necrosis, variable grades of fibrosis, and ultimately cirrhosis and an increased risk of hepatocellular carcinoma. Simple steatosis is largely benign and non-progressive whereas Nonalcoholic steatohepatitis (NASH) represents the more advanced stages of this disease, i.e. the 'inflammatory' component in addition to steatosis, which can lead to cirrhosis, liver failure, and hepatocellular carcinoma. (Bhatia et al, 2012) NAFLD is strongly associated with obesity, insulin resistance, hypertension, and dyslipidemia. And now it is regarded as the liver manifestation of metabolic syndrome.

### *2. Diagnosis of Nonalcoholic Fatty Liver Disease*

The early stages of NAFLD are often asymptomatic, slightly abnormal liver enzymes are usually the only clue pointing to the disease. Although alanine aminotransferase (ALT) levels have shown to be the best single biochemical correlates of hepatic steatosis, they do not distinguish between varying stages of NASH and can be normal in histologically severe disease. (Bhatia et al, 2012) Likewise, ultrasound imaging can only detect steatosis when 30% of the liver is affected. (Dowman et al, 2011) None of these non-invasive methods can detect inflammation and/or fibrosis, i.e. NASH. Accordingly, liver biopsy is at present the 'gold-standard' for diagnosing NAFLD and staging the degree of NASH and fibrosis by histological assessment, as well as monitoring disease progression. (McCullough, 2004) There are several ways to interpret liver biopsy. Despite the lack of consensus, Brunt et al (1999) proposed a three grade system (mild, moderate and severe) with four stages for NASH. Brunt's grading system is based on a combination of features including degrees of lobular involvement by steatosis, inflammation, ballooning degeneration, and portal inflammation; the pattern of progressive deposition of fibrosis: perivenular, perisinusoidal or bridging fibrosis, and eventually

cirrhosis forms the basis for staging fibrosis. (Fig.5) (Pinto and Camilo, 2004)

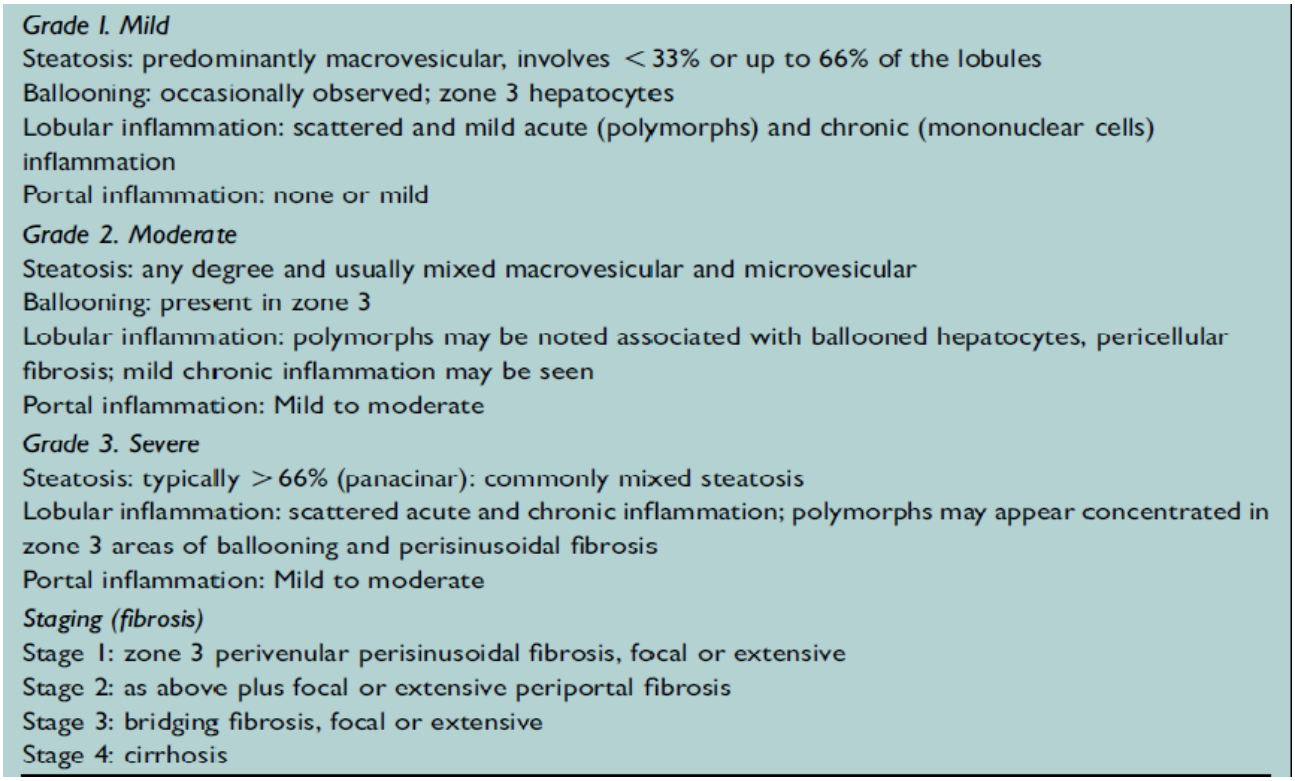



**Figure 5:** Grading and staging the lesions of NASH

### *3. Pathogenesis of fructose induced NAFLD*

The exact mechanism by which NAFLD/NASH is developed is not fully elucidated. However, research has attempted to identify the causes behind the development of this disease. According to the "two hits" theory proposed by Day and James in 1998, NAFLD is characterized by two steps of liver injury: intrahepatic lipid accumulation (hepatic steatosis) and hepatic inflammation. (Nomura et al, 2012)

Fat accumulates within the hepatocytes when the cellular fatty acid synthesis exceeds fatty acid degradation.( Basaranoglu et al, 2013) With fructose consumption, hepatocytic fatty acid input increased due to increased hepatic de-novo lipogenesis, inhibition of fatty acid β oxidation, impaired triglyceride clearance and reduced VLDL export. (Nomura et al, 2012)

As it is mentioned before, the bypass of the control step in fructose metabolism will lead to unregulated source of both glycerol-3-phosphate and acetylCoA leading to enhance lipogenesis. After fructose intake, triose phosphate (main lipogenic precursor) can be converted into pyruvate by pyruvate dehydrogenase (PDH) and further oxidized into $CO_2$ and $H_2O$ in the TCA cycle. (Nomura et al, 2012) When the liver mitochondria cannot metabolize excess of acetyl CoA substrate the extra substrate exits the mitochondria to cytosol in the form of citrate leading to de-novo lipogenesis. (Nomura et al, 2012)

De-novo lipogenesis is mediated by two chief proteins: carbohydrate response element binding protein (ChREBP) and sterol regulatory element binding protein 1c (SREBP-1C). (Strable et al, 2010) Fructose administration stimulates the activation of ChREBP and acts in synergy with SREBP-1C to boost the expression of lipogenic genes: acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and stearoyl enzyme-A desaturase-1(SCD 1). (Agius, 2012) (Figure: 5)

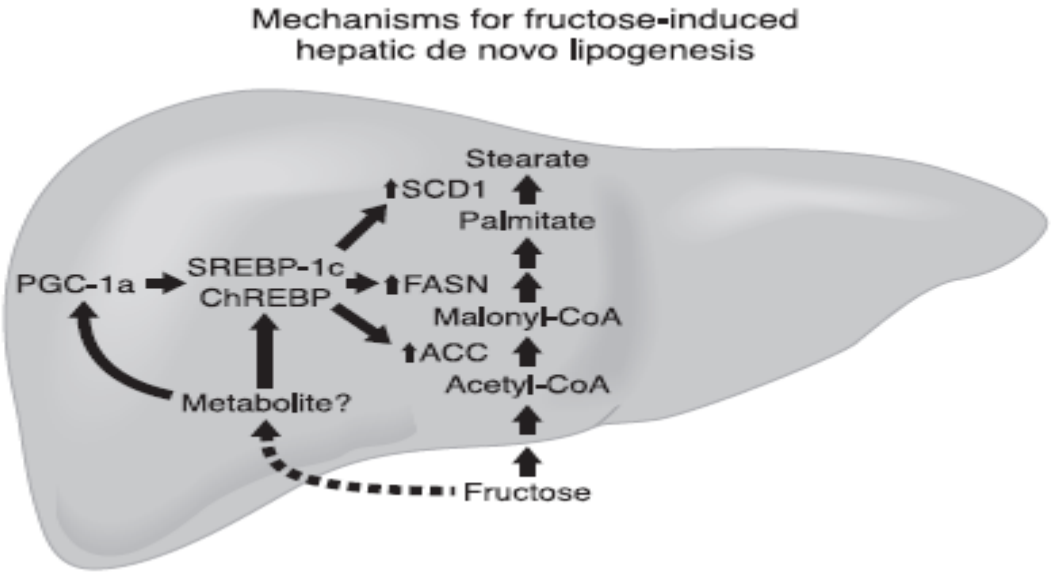



**Figure 6:** Mechanisms for fructose-induced hepatic de novo lipogenesis **(**Tappy et al,2010)

Another essential molecule associated with fructose regulation of lipids in the liver is peroxisome poliferator-activated receptor alpha (PPARα). Fructose-1-phosphate reduces hepatic PPARα mRNA levels which will lead to a reduction in the expression of fatty acid oxidation enzymes. (Nomura et al, 2012)

The initial step of de novo lipogenesis is the synthesis of malonyl-CoA which acts as a potent inhibitor of carnitine palmitoyl transferase-1. Therefore, de novo lipogenesis and lipid oxidation are simultaneously and inversely regulated by fructose in liver cells. (Le

etal, 2009) Accordingly, Le et al (2009) showed that high fructose diet stimulates de novo lipogenesis in humans, while reducing whole body lipid oxidation.(Le et al,2009)

The second hit is proposed to be inflammation caused by oxidative stress associated with lipid peroxidation, cytokine activation, reactive oxygen species (ROS) and endogenous toxins of fructose metabolites. (Nomura et al, 2012) Elevated dietary fructose intake encourages bacterial overgrowth in the small intestine accompanied by intestinal permeability. This increases endotoxins levels such as bacterial lipopolysaccharide in the portal vein. .( Yilmaz, 2012) Endotoxins induce the expression of the toll-like receptor 4 (TLR4) (endotoxin receptor) in the liver. The binding of lipopolysaccharides to TLR4 in the hepatocytes induces lipid peroxidation and the production of reactive oxygen species (ROS). Increased ROS formation can mediate the release of proinflammatory cytokines such as TNFα. TNFα can then cause insulin resistance in hepatocytes, which in turn may alter the secretion of hepatic triglycerides. Therefore increased ROS formation has been frequently stated as a major contributor to the second hit mechanism of NAFLD. (Spruss et al, 2009) (Fig.6) Spruss et al. confirmed the hypothesis that the onset of fructose-induced NAFLD in mice is related to intestinal bacterial overgrowth and increased intestinal permeability, which eventually lead to an endotoxin-dependent activation of hepatic Kupffer cells. (Yilmaz, 2012)

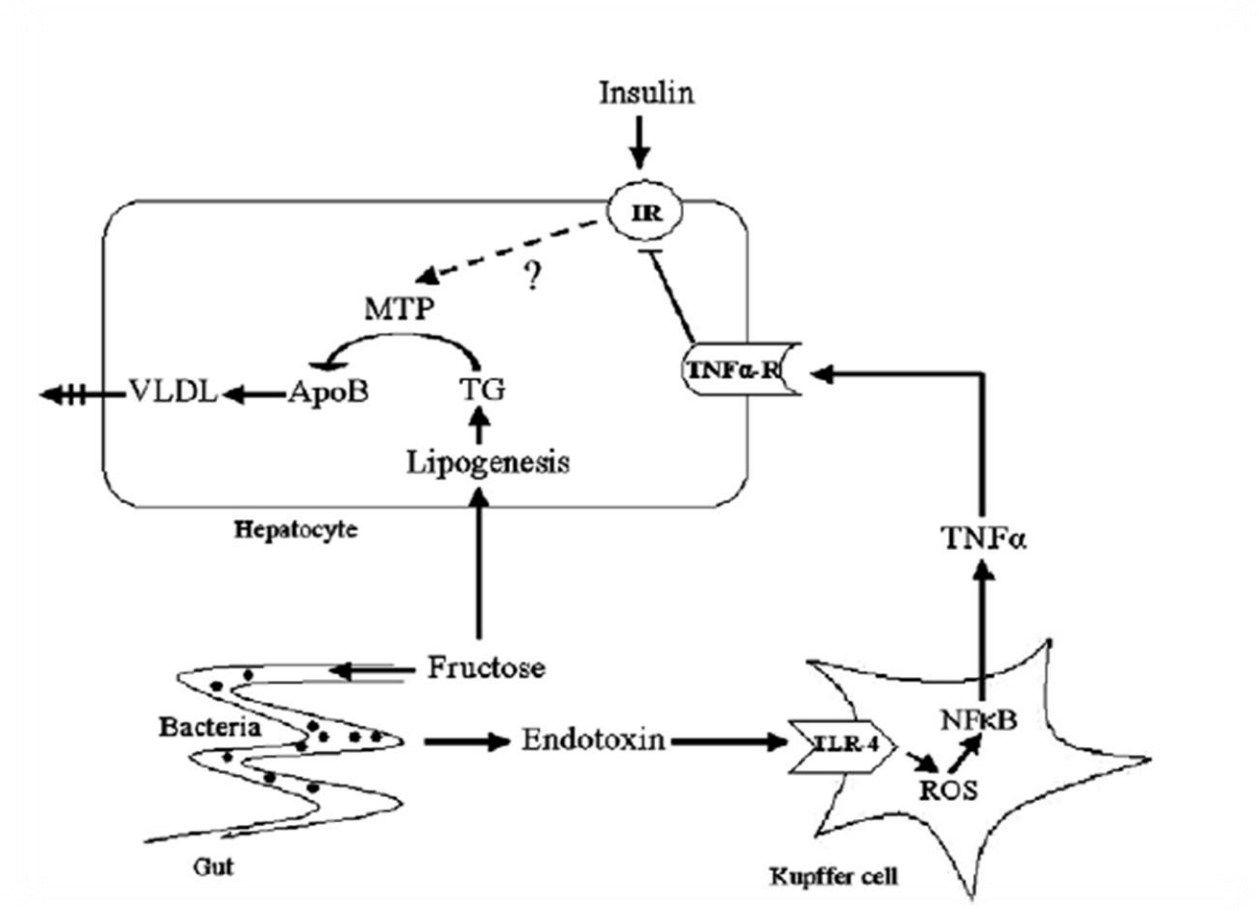



**Figure 7:** Possible molecular mechanism involved in the development of fructose-induced NAFLD (Spruss et al, 2009)

## 4. Fructose loading in relation to liver ATP and NAFLD

Besides the proposed "two hits" hypothesis, recent studies also suggest that ATP depletion and uric acid formation could be involved in the development and progression of NAFLD. During the metabolism of fructose, there was rapid loss of total adenine nucleotides. (Cannon et al, 1991) The rapid phosphorylation of fructose by fructokinase produces fructose 1-phosphate and consumes ATP as a phosphorus provider. (Cannon et al, 1991) Therefore, high fructose intake leads to a rapid decrease in cellular ATP and Pi. Pi is trapped in fructose-1-phosphate or reutilized in the formation of new ATP (Cannon et al, 1991). Cannon et al (1991) have shown that incubation of hepatocytes with 50mM fructose caused intracellular ATP to fall from approximately 5.0 to 0.8 mM due to the depletion of Pi. (Cannon et al, 1991) The fall in intracellular phosphate levels resulted in the stimulation of adenosine monophosphate (AMP) deaminase. AMP deaminase is thought to be responsible for the initial irreversible degradation of AMP to IMP. (Van den berghe et al, 1977) Consequently the increased stimulation of AMP deaminase pushes AMP toward the production of uric acid as opposed to the regeneration of ATP by AMP kinase. It was shown that high plasma uric acid may be involved in the development of insulin resistance through the inhibition of NO synthase. (Tappy et al, 2010) Also, it was suggested that uric acid may itself have proinflammatory and oxidative effects.

Impaired hepatic energy homeostasis (i.e., ATP depletion) may also explain the proposed associations of increased fructose consumption with increased hepatic inflammation; failure to provide ATP for the triglyceride synthesis may fail to transform toxic free fatty acids to a

safer form of lipids (i.e., triglycerides) and exacerbate lipotoxicity. (Abdelmalek, et al 2010).

Furthermore, some studies showed that cellular ATP depletion could interfere in apoB secretion. Apoprotein B100 like other secretory proteins are synthesized and cotranslationally translocated through the membrane of the ER into its lumen. (Zhou et al, 1995) Apoprotein B100 is a very large hydrophobic protein. Its secretion is regulated post-translationally rather than at the transcriptional or translational level. The key step in this post-translational regulation appears to be the translocation of newly synthesized apoB across the ER membrane. Inefficient translocation allows some portion of apoB to be exposed to the cytosol and this renders nascent apoB susceptible to degradation by protease.

Newly synthesized apoB transiently interacts with heat shock protein Hsp 70. This protein not only assists proteins as they mature into their final conformation, but also participates in apoB translocation across the ER membrane and in its degradation by proteasome.

Hsp 70 protein contains two domains: an amino-terminal ATPase and a carboxy-terminal peptides binding domain. ATP binding induces Hsp70 conformational changes that lead to dissociation of Hsp70 from nascent ApoB. In the event of ATP depletion the dissociation of Hsp70 from apoB does not occur. The association of Hsp70 with apoB maintains this secretory protein in a transmembrane position. In this position, apoB appears to be very sensitive to proteolysis. (Zhou et al, 1995)

Many studies suggested that TG availability is a major factor in the post –translational regulation of apoB secretion. ApoB is assembled with lipids and secreted as very low density lipoprotein. The secretion of apoB requires its assembly with lipids. The initial assembly of apoB with lipids is cotranslational although production of mature VLDL particles may require post-translational lipid addition. (Ingram, 1996) It has been suggested that the translocation of apoB across the ER membrane is coupled to its assembly with lipids. Then, apoB that fails to engage in lipoprotein assembly is inefficiently translocated and undergoes transmembrane integration leading to its intarcellular degradation by proteasome. (Fisher et al, 1997)

Triglyceride (TG) which is synthesized exclusively by the ER can enter two major pathways: the secretory pathway in which assembly into VLDL occurs (in Golgi apparatus) and a cytoplasmic storage pathway outside of the secretory system. (Bambergeret al, 1990) Studies showed that the rapid movement of TG from the ER to Golgi appears to be ATP-dependent. Therefore in the case of ATP depletion the movement of TG into Golgi decreases while the movement of TG from ER into cytoplasmic triacylglycerol-rich vesicles TGRVs continues (storage pathway). Thus the assembly and the secretion of VLDL are decreased and the TG will accumulate inside the cells causing fatty liver. ( Bambergeret al, 1990)

As a result ATP depletion and uric acid formation in hepatocytes are suggested to be involved in the progression of NAFLD.

At this point, attention was raised on the role of phosphorus in fructose-induced fatty liver disease and to the extent the manipulation of phosphorus can affect this disease. The mechanisms underlying fructose induced fatty liver diseases remain poorly understood and might be related to the phosphorus concentration inside the hepatocytes. Therefore, manipulating the phosphorus content of the diet and its concentration inside the cells could be of great influence on the progression of non-alcoholic fatty liver disease. The present study was designed to investigate the role of phosphorus in the development of NAFLD.

# CHAPTER III

# MATERIALS AND METHODS

## A. Experimental procedure

### *1. Animal housing:*

Forty- three male Sprague-Dawley rats weighing between 160-250 g were used. Rats were housed individually in wire-bottomed cages to facilitate collection of spilled food. Room temperature was maintained at 22±1°C with 12:12 dark-light cycle. The rats had free access to food and water and were fed *ad libitum* on an adaptation diet (control diet) for one week to familiarize them to the environment and diet.

### *2. Experimental Diets:*

The composition of the control diet (and the adaptation period) is shown in Table 1. The experimental diets were prepared using the same ingredients in different proportions, with the exception of the mineral mix which was phosphorus free.

Potassium phosphate was used in different proportions to manipulate the phosphorus content of the different diets. The composition of the vitamin and mineral mixes are presented in the Appendix.

***3. Experimental design:***

This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the medical school at the American University of Beirut (AUB).

Following adaptation, rats were randomly divided into five groups, four of which were placed on a high fructose diet (60%) with different levels of phosphorus except for group 1 (control group) which received a diet with 30% starch and 30% sucrose.

- Group 1: Control: 30% starch and 30% sucrose / 0.3% P
- Group 2: High Fructose/ 0.15% Phosphorus
- Group 3: High Fructose/ 0.3% Phosphorus
- Group 4: High Fructose/ 0.6% Phosphorus
- Group 5: High Fructose/ 0.9% Phosphorus

Rats were kept on the experimental diet for 10 weeks. Food intake and body weight were measured twice per week. Food intake was measured by subtracting the weight of the pot from the previous weight taking into account food spillage.

On the final day, rats were fasted overnight and the following morning at 9:00 a.m they were sacrificed by decapitation. Blood was collected from the neck vessels in EDTA tubes and was placed on ice. Liver, kidney and epididymal fat pads (EFP) were weighed and immediately excised and frozen in liquid nitrogen, then stored at -80°C till analysis. Sections from the liver of each animal were put in formaldehyde and in saline for liver biopsy. Blood samples were then centrifuged at 3500 rpm for 15minutes at 3°C within the following 1 hour. Plasma aliquots were stored at -80°C till analysis

**Table 1:** Diet composition

| Diet (g/100g) | Adaptation diet | Group 1 (control) | Group 2 | Group 3 | Group 4 | Group 5 |
|---|---|---|---|---|---|---|
| Protein (casein) | **20%** | **20%** | **20%** | **20%** | **20%** | **20%** |
| DL-Methionine | **0.2%** | **0.2%** | **0.2%** | **0.2%** | **0.2%** | **0.2%** |
| Fat (corn oil) | **10%** | **10%** | **10%** | **10%** | **10%** | **10%** |
| Fructose | **-** | **-** | **60%** | **60%** | **60%** | **60%** |
| Starch | **30%** | **30%** | **-** | **-** | **-** | **-** |
| Sucrose | **30%** | **30%** | **-** | **-** | **-** | **-** |
| Mineral mix | **3.5%** | **3.5%** | **3.5%*** | **3.5%*** | **3.5%*** | **3.5%*** |
| Vitamin mix | **1%** | **1%** | **1%** | **1%** | **1%** | **1%** |
| Cellulose | **7%** | **7%** | **7%** | **7%** | **7%** | **7%** |
| Potassium phosphate ($KH_2PO_4$)(g) | **-** | **-** | **-** | **0.66 (0.15%)** | **1.98 (0.45%)** | **3.295 (0.75%)** |
| Total Phosphorus | **0.3%** | **0.3%** | **0.15%** | **0.3%** | **0.6%** | **0.9%** |

**** Phosphorus free***

## B. Blood analysis

### *1. Plasma Insulin*

Insulin was determined by enzyme immunoassay using an insulin ELISA kit provided by DiaMetra, Milano, Italy.

### *2. Plasma glucose, Triglyceride, total cholesterol, HDL,LDL and total phosphorus*

Plasma glucose, triglycerides, total cholesterol, HDL, LDL and total phosphorus were determined using an enzymatic colorimetric method on the Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Johnson & Johnson, New York).

### *3. Plasma ALT and AST*

Plasma ALT and AST were determined in the chemistry lab at the AUBMC. Samples were run on Cobas 6000 instrument based on the following principle: The oxacids formed in the transaminase reaction were measured indirectly by enzymatic reduction to the corresponding hydroxyacids, and the accompanying change in NADH concentration was monitored spectrophotometrically.

## C. Liver Analysis

### 1. *Hepatic Fat Extraction.*

Liver lipids were extracted using the method of Folch et al. (1957). The liver portion designated for fat extraction was weighed alone and put in a centrifuge tube. The

centrifuge tube including the liver portion was weighed then was freeze dried. After freeze drying, the liver was crushed in the tube using a glass rod till it became powder. Then 25 ml of chloroform:methanol (2:1volume) were added to the tube. The liver and the chloroform:methanol were mixed with a vortex and shaken in an orbital shaker for 15-20 minutes. The mixture was then for 15 minutes at 3500 rpm and the top layer containing the fat was discarded leaving what was left in the tubes to dry in the fume hood over the weekend. After the weekend, the centrifuge tube + remaining residues were weighed.

**2. *Liver biopsy***

Liver biopsies were done by a pathologist from the AUBMC. Sections from the liver of each animal were stained with hematoxylin and eosin (H&E) and oil red (O) for the evaluation of necro-inflammatory grading and fatty droplets.

a- Histopathologic Examination

Rat liver tissue was processed into 3-4 μm thick formalin-fixed paraffin embedded tissue sections and stained with hematoxylin and eosin (H&E). Histopathologic examination consisted of assessing steatosis grade and distribution with a score=0 (<5%); score=1 (5%-33%); score=2 (>33%-66%) and score=3 (>66%). Location was defined as steatosis distribution with a score=0 (zone 3); score=1 (zone 1); score=2 (azonal); or score=3 (panacinar). Microvesicular steatosis was recorded as either score=0 (not present) or score=1 (present). Lobular inflammation was semiquantified according to a score=0 (< 2

foci per 200x field); score=1 (2-4 foci per 200x field); or score=3 (>4 foci per 200x field). (Kleiner et al, 2005)

b- Oil Red O (ORO) Examination

ORO was performed according to the described protocol (Melhem et al. ,2013). Briefly fresh frozen rat liver tissue was embedded into cryomolds and sectioned into 5 µm sections on a cryostat (Leica). Sections were then stained in ORO and semiquantified using image j software (http://rsbweb.nih.gov/ij). Tissue sections were imaged at five high-power (400x) fields and converted to 8-bit grayscale images; This was followed by an image threshold predefined according to a rat liver section negative for steatosis and microvesicular steatosis on H&E and ORO staining. Subsequently image analysis for ORO surface area staining was determined.

**D. Statistical Analysis**

Data are expressed as means ± SEM of all values. Data analysis was performed using the MINITAB 16 software program. Results were analyzed by a one way analysis of variance (ANOVA), and specific comparisons were made between each of the five groups and among the high fructose groups only using Fisher's pairwise comparisons. A probability of less than 0.05 was considered to be significant

# CHAPTER IV

# RESULTS

## A- Body Weight and Food intake

### *1. Weight and weight gain*

The initial mean weight and the final mean weight of each group were calculated and documented in Table 2. At the beginning, rat weights were similar in all five groups. At the end of the ten-week experiment, when comparing all groups control group (starch+sucrose) showed significantly higher mean weight than the high fructose groups (P=0.018). However, when comparing high fructose groups no significant difference of body weight was observed among them. This result revealed that phosphorus manipulation did not affect body weight (P=0.349)

Weight gain data were expressed as g/day. By comparing all groups the result showed that Control group had a significantly higher mean of daily weight gain than the means of the other groups. (P=0.002) However the average daily weight gain during the study was not affected by phosphorus intake since high fructose groups tended to have similar daily weight gain. (P=0.475)

### *2. Food intake and Energy intake*

The average food intake of each group was calculated and summarized in Table2. Data were expressed as g/d. when comparing all groups the result showed a significant difference between the five groups. (P=0.044). The average food intake of the HF/ 0.3%P was lower than the average of other groups. In addition the difference in food intake remains significant even when comparing the high fructose groups alone. (P=0.026)

**Table 2:** Body weight, food intake/d, food efficiency and weight gain/d of male Sprague-Dawley rats maintained for 10 weeks on high-fructose diets with different levels of phosphorus. Results are expressed as mean ± SE

| | Control n= 9 | HF/0.15%P n= 9 | HF /0.3% P n=8 | HF/0.6%P n=9 | HF /0.9%P n= 8 | Anova P-value | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High Fructose groups |
| Initial body weight (g) | 237.7±9.99 | 237.53±8.83 | 235.42±8.57 | 238.46±8.88 | 240.5±9.91 | 0.997 | 0.984 |
| Final Body weight (g) | $560.9\pm18.0^{a}$ | $510.5 \pm 14.7^{ab}$ | $468.0\pm24.0^{b}$ | $503.7\pm 18.5^{b}$ | $506.0\pm13.3^{b}$ | 0.018 | 0.349 |
| Food Intake (g/day) | $26.46\pm1.12^{a}$ | $25.344^{\alpha} \pm0.955^{ab}$ | $22.64^{\beta} \pm1.05^{b}$ | $26.433^{\alpha} \pm0.601^{a}$ | $26.57^{\alpha} \pm1.16^{a}$ | 0.044 | 0.026 |
| Weight gain (g/day) | $5.000 \pm 0.289^{a}$ | $4.000 \pm0.167^{b}$ | $3.500 \pm0.378^{b}$ | $3.889 \pm0.200^{b}$ | $3.875 \pm0.125^{b}$ | 0.002 | 0.475 |
| Food efficiency (g/100Kcal) | $4.75\pm0.14^{a}$ | $4.02\pm0.19^{b}$ | $3.85\pm0.28^{b}$ | $3.72\pm0.20^{b}$ | $3.73\pm0.20^{b}$ | 0.005 | 0.763 |

$^{a, b}$*: Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for all groups.*

$^{\alpha,\beta}$ *: Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for fructose groups .*

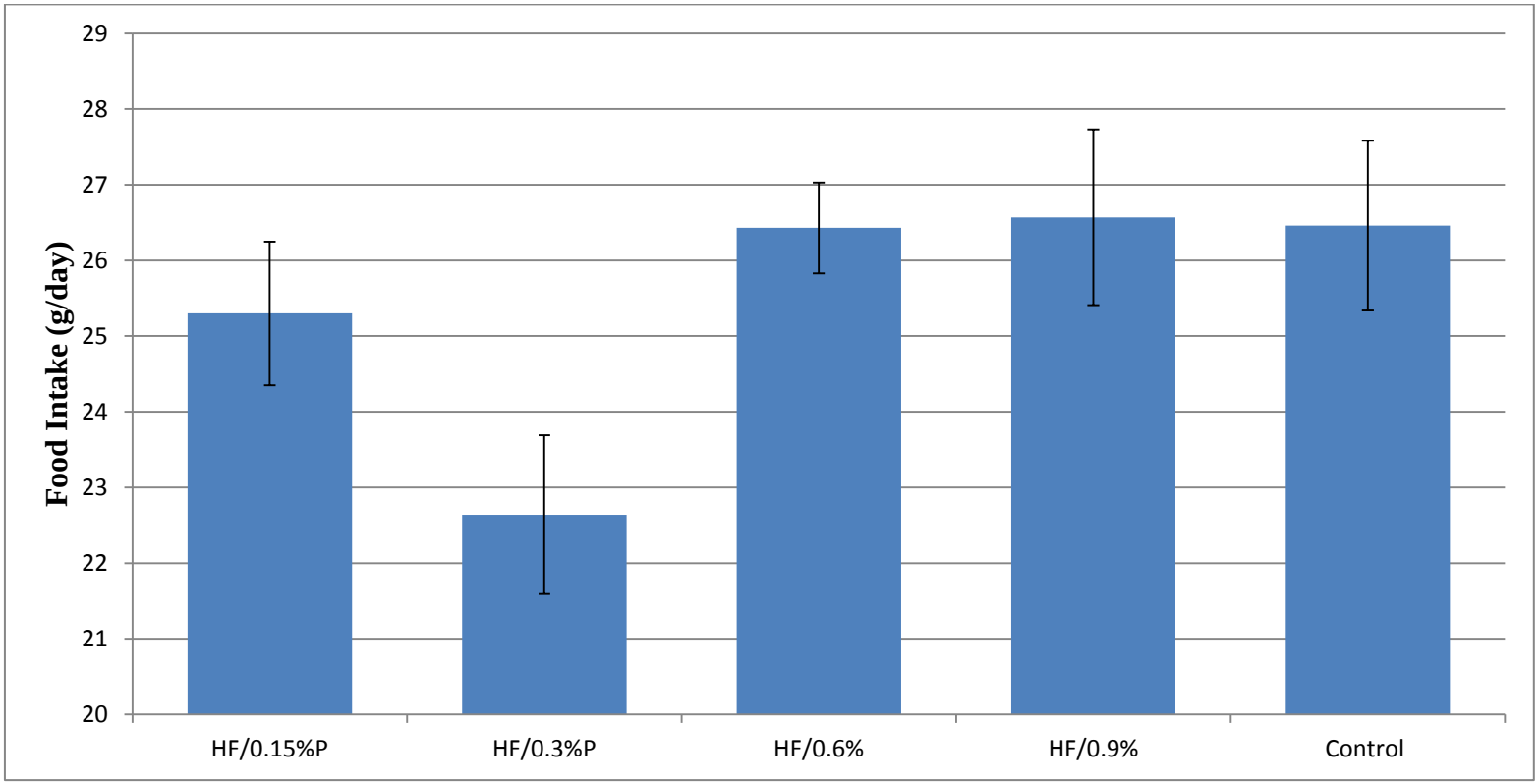



**Figure 8:** Food intake (g/day) of male Sprague-Dawley rats maintained for 10 weeks on high-fructose diets with different levels of phosphorus

**B. Organ weight**

***1. Liver weight:***

Anova of all groups showed that the HF/0.3% P had lower liver weight than the other groups but this difference did not reach the significance level P=0.085

In addition, Anova of high fructose groups showed that HF/0.6%P had significantly higher liver weight than the other groups. (P=0.048)

***2. Kidney weight:***

The result of comparing all groups showed that the HF/0.9% P had significantly higher kidney weight per 100g body weight than the other groups (P=0.004). This result revealed that the kidney weight/100gBW increases with the increased of the percentage of phosphorus intake. However, when comparing high fructose groups, no significant differences in kidney weights were found. (P=0.206)

**3. Epididymal Fat Pads Weight:**

When comparing all groups, the HP/0.3%P had lower Epididymal Fat Pads Weights per 100g body weight than the other groups but this difference did not reach the significance level P=0.158. By comparing the high fructose groups it was revealed that phosphorus manipulation did not affect Epididymal Fat Pads Weight since no significant difference was observed between the high fructose groups (P =0.820).

**Table 3*:*** liver weight, kidney weight and EPD weight of male Sprague-Dawley rats maintained for 10 weeks on high-fructose diets with different levels of phosphorus. Results are expressed as mean ± SE

| | Control n= 9 | HF/0.15%P n= 9 | HF/0.3% P n=9 | HF/0.6%P n=8 | HF/0.9%P n= 8 | Anova P- value | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High Fructose groups |
| Liver weight (g) | $15.99 \pm 0.94^{a}$ | $14.86^{\alpha\beta} \pm 0.652^{ab}$ | $13.56^{\beta} \pm 0.86^{b}$ | $16.40^{\alpha} \pm 0.59^{a}$ | $14.91^{\alpha\beta} \pm 0.59^{ab}$ | 0.085 | 0.048 |
| Liver weight/100g | $2.84 \pm 0.11^{b}$ | $2.90 \pm 0.08^{b}$ | $2.895 \pm 0.115^{b}$ | $3.281 \pm 0.16^{a}$ | $2.94 \pm 0.09^{b}$ | 0.066 | 0.070 |
| Kidney weight (g) | $3.39 \pm 0.145^{ab}$ | $3.79 \pm 0.317^{a}$ | $3.106 \pm 0.153^{b}$ | $3.637 \pm 0.154^{ab}$ | $3.828 \pm 0.143^{a}$ | 0.083 | 0.088 |
| Kidney weight/ 100g | $0.60 \pm 0.018^{c}$ | $0.74 \pm 0.0542^{ab}$ | $0.66 \pm 0.0123^{bc}$ | $0.72 \pm 0.0130^{ab}$ | $0.75 \pm 0.0146^{a}$ | 0.004 | 0.206 |
| EPD weight (g) | $12.50 \pm 1.68^{a}$ | $8.86 \pm 1.09^{b}$ | $7.37 \pm 0.869^{b}$ | $8.50 \pm 1.25^{b}$ | $8.195 \pm 0.960^{b}$ | 0.048 | 0.791 |
| EPD weight /100g | $2.183 \pm 0.249^{a}$ | $1.717 \pm 0.191^{ab}$ | $1.463 \pm 0.182^{b}$ | $1.653 \pm 0.225^{ab}$ | $1.604 \pm 0.162^{ab}$ | 0.158 | 0.820 |

$^{a, b}$*: Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for all groups*

$^{\alpha,\beta}$ : *Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for fructose groups .*

**C. Plasma *ALT and AST:***

Mean ALT and AST concentrations are shown in Table 4. Both Anova of all groups and Anova of high fructose groups showed no significant difference in each parameter among the groups.

**D. Lipid profile:**

Mean serum HDL, LDL, total cholesterol and TG concentrations are shown in Table 5. No significant difference was observed in each parameter among the groups. Phosphorus manipulation did not show any effect on lipid profile of rats.

**Table 4:** Plasma ALT and AST of male Sprague-Dawley rats maintained for 10 weeks on high-fructose diets with different levels of phosphorus. Results are expressed as mean ± SE

| | Control n= 9 | HF/0.15%P n= 9 | HF/ 0.3%P n=9 | HF/0.6%P n=8 | HF/0.9%P n= 8 | Anova P- value | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High fructose groups |
| AST (IU/L) | 158.6±14.1 | 184.2 ± 37.6 | 179.3 ±26.3 | 139.9 ±20.3 | 207.1 ±59.0 | 0.691 | 0.651 |
| ALT (IU/L) | 44.78±3.87 | 56.9 ± 11.9 | 57.50 ± 7.83 | 51.67 ±9.17 | 60.0 ± 18.8 | 0.877 | 0.970 |

**Table 5:** HDL, LDL, total cholesterol and TG concentrations of male Sprague-Dawley rats maintained for 10 weeks on high-fructose diets with different levels of phosphorus. Results are expressed as mean ± SE

| | Control n= 9 | HF/0.15%P n= 9 | HF /0.3%P n=9 | HF/0.6%P n=8 | HF /0.9%P n= 8 | Anova P- value | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High fructose groups |
| HDL (mg/dl) | 53.44 ±2.8 | 46.33 ± 4.90 | 50.63 ± 4.48 | 49.67 ± 3.42 | 53.13 ± 4.26 | 0.714 | 0.73 |
| LDL (mg/dl) | 32.00±3.03 | 26.44 ± 3.95 | 26.00 ± 5.25 | 29.00 ± 5.02 | 24.88 ± 2.94 | 0.752 | 0.92 |
| Cholesterol (mg/dl) | 95.22±6.99 | 80.67±9.06 | 82.13± 8.78 | 88.33 ±7.97 | 86.00± 7.13 | 0.716 | 0.906 |
| TG (mg/dl) | 49.6±11.7 | 39.56±6.70 | 28.88 ±2.18 | 46.78± 5.80 | 39.50 ± 6.10 | 0.345 | 0.186 |

**E. Plasma glucose and insulin**

Mean glucose and insulin concentrations are shown in Table 6. Blood glucose showed no significant difference between the groups. Alternatively, when comparing all groups, the concentration of fasting blood insulin in the control group was significantly higher than the concentration of the other groups (P=0.038) However, Anova of high fructose groups did not show significant difference in fasting blood insulin among the high fructose groups. (P=0.078)

**F. Plasma Total phosphorus**

As shown in Table 6, No significant difference was noted across groups regarding total phosphorus levels. $P_{\text{all groups}}=0.583$, $P_{\text{high fructose groups}}=0.44$)

**Table 6:** Insulin, glucose and total phosphorus concentrations of male Sprague-Dawley rats maintained for 10 weeks on high-fructose diets with different levels of phosphorus. Results are expressed as mean ± SE

| | Control n= 9 | HF/0.15%P n= 9 | HF/ 0.3%P n=9 | HF/0.6%P n=8 | HF /0.9%P n= 8 | Anova P- value | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High fructose groups |
| Insulin ( µIU/ml) | $18.01\pm5.08^{a}$ | $7.55^{\beta}\pm1.78^{b}$ | $6.02^{\beta}\pm1.47^{b}$ | $12.09^{\alpha}\pm2.12^{ab}$ | $8.66^{\alpha\beta}\pm2.12^{b}$ | 0.038 | 0.078 |
| Glucose (mg/dl) | 99.89±5.79 | 104.56±9.33 | 100.13±3.44 | 116.78±5.74 | 109.88±6.21 | 0.315 | 0.343 |
| Total Phosphorus (mg/dl) | 5.31±0.316 | 5.800±0.439 | 5.075±0.171 | 5.467±0.317 | 5.438±0.160 | 0.583 | 0.44 |

$^{a, b}$*: Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for all groups*

$^{\alpha,\beta}$ : *Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for fructose groups .*

## G. Liver analysis

### *1. Liver fat content:*

No significant difference was seen between the groups in liver fat content. Liver fat content was not significantly affected by high fructose intake. ($P_{\text{all groups}} = 0.15$) Also, liver fat content was not significantly affected by the level of phosphorus intake. ($P_{\text{high fructose groups}} = 0.36$)

### *2. Histology*

The results of biopsy were summarized in Table 8. No significant difference was noted across groups regarding steatosis grade, portal and lobular inflammation and ballooning.

However Anova of all groups and Anova of high fructose groups showed statistical significant among groups in which the HF/0.15%P group showed significantly lower microvesicular steatosis than the other groups ($P_{\text{all groups}} = 0.003$, $P_{\text{high fructose groups}} = 0.001$). The results revealed that microvesicular steatosis increased with the increase of the percentage of phosphorus intake (Fig.8).

ORO examination was used to evaluate hepatic lipid accumulation. When grouping the data according to all groups, no significant change was observed in hepatic lipid accumulation. Furthermore , Anova of high fructose groups showed that the

HF/0.15% P had lower fat accumulation than the other groups but this difference did not reach the significance level $P_{\text{high fructose groups}} = 0.359$ (Fig.9)

The zonal location of the steatosis was also analyzed. Anova of all groups and Anova of High fructose groups showed significant change in steatosis location. A zone 3 distribution of steatosis was the most common pattern in HF/0.15%P and HF/0.3%P groups while zone 1 distribution of steatosis dominated the HF/0.6%P and HF/0.9%P groups. In this study, azonal pattern was very rare, portal inflammation, fibrosis, liver cell ballooning, Mallory hyaline bodies and glycogenated nuclei were all absent.

**Table 7**: Liver fat contents of male Sprague-Dawley rats maintained for 10 weeks on high-fructose diets with different levels of phosphorus. Results are expressed as mean ± SE

| | Control n= 9 | HF/0.15%P n= 9 | HF / 0.3% P n=8 | HF/0.6%P n=9 | HF /0.9%P n= 8 | Anova P-value | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High fructose groups |
| Liver fat content (% wet weight) | 2.45±0.30 | 2.25±0.15 | 2.61±0.22 | 2.17±0.21 | 2.49±0.16 | 0.5 | 0.365 |
| Liver fat content (% Dry weight) | 4.1±0.50 | 4.4±0.41 | 5.2±0.47 | 3.9±0.48 | 5±0.51 | 0.3 | 0.167 |

**Table 8:** liver histology of male Sprague-Dawley rats maintained for 10 weeks on high fructose diets with different levels of phosphorus. Results are expressed as mean ± SE

| | Control n= 9 | HF/0.15%P n= 9 | HF / 0.3% P n=8 | HF/0.6%P n=9 | HF /0.9%P n= 8 | Anova P- value | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High fructose groups |
| Steatosis Grade | 0.78 ±0.40 | 0.11±0.11 | 0.37±0.37 | 0.67 ±0.33 | 0.37 ± 0.18 | 0.540 | 0.533 |
| Steatosis Location | $1.33 \pm 0.44^{a}$ | $0.22^{\beta} \pm 0.22^{b}$ | $0.75^{\alpha\beta} \pm 0.36^{ab}$ | $1.67^{\alpha} \pm 0.37^{a}$ | $1.50^{\alpha} \pm 0.38^{a}$ | 0.041 | 0.015 |
| Microvesicular steatosis | $0.67 \pm 0.17^{ab}$ | $0.22^{\gamma} \pm 0.14^{c}$ | $0.50^{\beta\gamma} \pm 0.19^{bc}$ | $1.00^{\alpha} \pm 0.0^{a}$ | $0.87^{\alpha\beta} \pm 0.12^{ab}$ | 0.003 | 0.001 |
| Lobular inflammation | $0.50 \pm 0.19^{ab}$ | $0.67 \pm 0.17^{a}$ | $0.12 \pm 0.12^{b}$ | $0.56 \pm 0.17^{ab}$ | $0.50 \pm 0.27^{ab}$ | 0.349 | 0.232 |
| Portal inflammation | 0 | 0.11 ±0.11 | 0 | 0 | 0.12± 0.12 | 0.551 | 0.576 |
| Ballooning | $0.44 \pm 0.24^{a}$ | $0.11 \pm 0.11^{ab}$ | $0^{b}$ | $0.11 \pm 0.11^{ab}$ | $0^{b}$ | 0.141 | 0.627 |
| OilredImage analysis | 3293 ± 1130 | $1319^{\alpha} \pm 1141$ | $4622^{\alpha\beta} \pm 2854$ | $5480^{\alpha} \pm 1567$ | $4363^{\alpha\beta} \pm 1079$ | 0.431 | 0.359 |

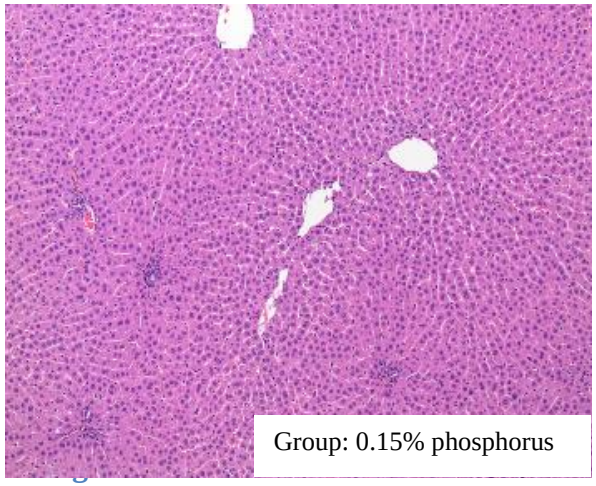
*$^{a, b}$: Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for all groups*

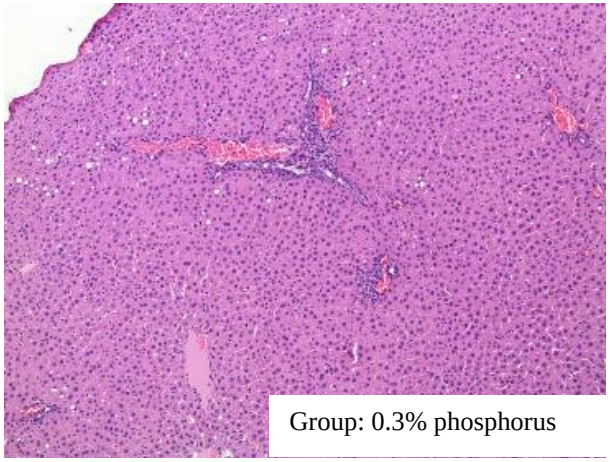
*$^{\alpha\beta\gamma}$: Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for fructose groups*

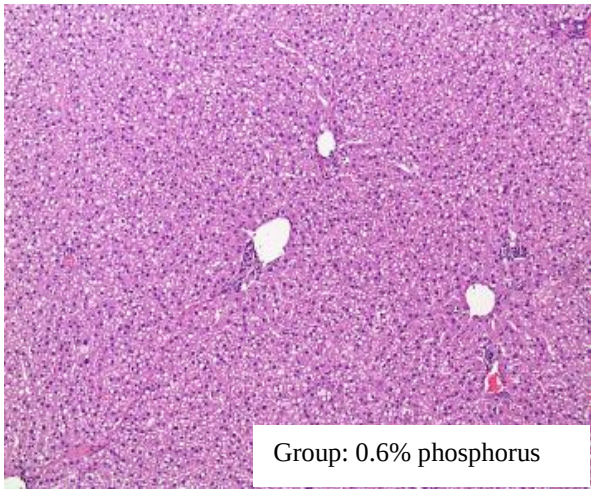
**Table 9:** Steatosis grade, microvesicular steatosis and steatosis location of male Sprague-Dawley rats maintained for 10 weeks on high fructose diets with different levels of phosphorus.

| | Control n=9 | HF/0.15%P n=9 | HF/0.3%P n=8 | HF/0.6%P n=9 | HF/0.9%P n=8 | P-Anova | |
|---|---|---|---|---|---|---|---|
| | | | | | | All groups | High fructose groups |
| **Steatosis grade** | | | | | | | |
| Grade: 0 | 6/9 (67%) | 8/9(89%) | 7/8 (87.5%) | 5/9 (56%) | 5/8(62.5%) | 0.443 | 0.296 |
| Grade: 1 | 0 (0%) | 1/9(11%) | 0 (0%) | 3/9(33%) | 3/8 (37.5%) | 0.089 | 0.195 |
| Grade: 2 | 2/9 (22%) | 0(0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0.094 | - |
| Grade: 3 | 1/9 (11%) | 0(0%) | 1/8(12.5%) | 1/9 (11%) | 0(0%) | 0.74 | 0.576 |
| Total n of rats with any stage of Steatosis (%) | 3/9(33%) | 1/9(11%) | 1/8(12.5%) | 4/9(44%) | 3/8(37.5%) | | |
| **Microvesicular steatosis** | | | | | | | |
| Absent | 3/9(33%) | 7/9(78%) | 4/8(50%) | 0(0%) | 1/8(12.5%) | | |
| Present | 6/9(67%)$^{ab}$ | 2/9(22%)$^{c}$ | 4/8(50%)$^{bc}$ | 9/9(100%)$^{a}$ | 7/8(87.5%)$^{ab}$ | 0.008 | 0.003 |
| **Steatosis location** | | | | | | | |
| Zone3 | 3/9(33.3%)$^{b}$ | 8/9(88.9%)$^{a}$ | 4/8(50%)$^{ab}$ | 1/9(11.1%)$^{b}$ | 1/8 (12.5%)$^{b}$ | 0.002 | 0.001 |
| Zone 1 | 3/9(33.3%)$^{ab}$ | 0 (0%)$^{b}$ | 3/8(37.5%)$^{ab}$ | 4/9(44.4%)$^{a}$ | 4/8(50%)$^{a}$ | 0.200 | 0.108 |
| Azonal | 0(0%) | 1/9(11.1%) | 0(0%) | 1/9(11.1%) | 0(0%) | 0.603 | 0.627 |
| panacinar | 3/9(33.3%) | 0(0%) | 1/8(12.5%) | 3/9(33.3%) | 3/8(37.5%) | 0.287 | 0.188 |

$^{ab}$: *Values in the same row with different superscripts are significantly different based on Fisher's pairwise comparison (P<0.05) anova for all groups*



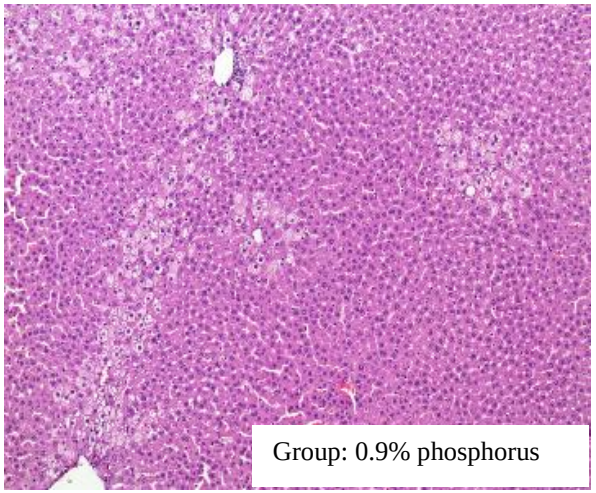
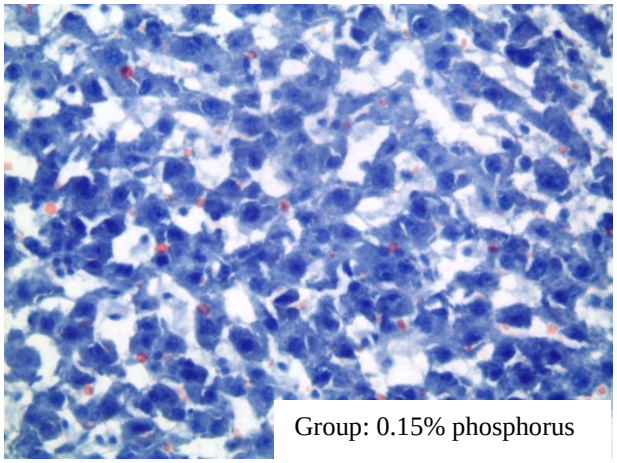
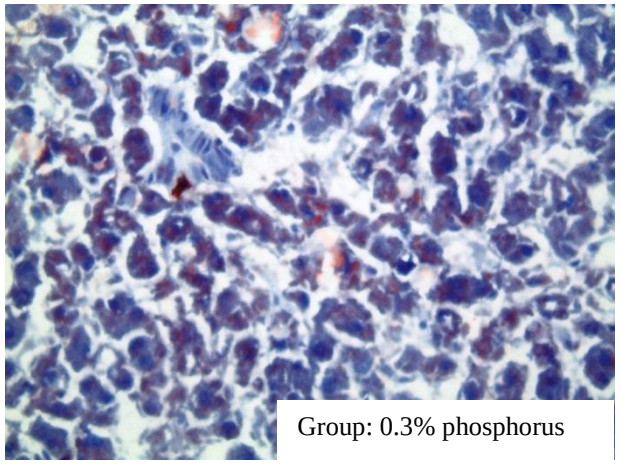
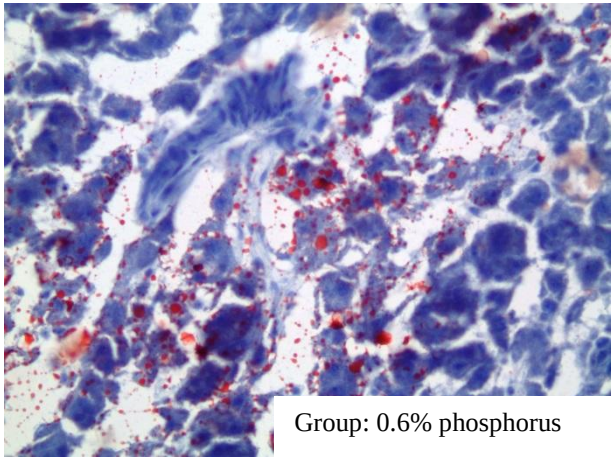




**Figure 9:** Representative images of H and E staining of liver sections from male Sprague-Dawley rats maintained for 10 weeks on high fructose diets with different level of phosphorus



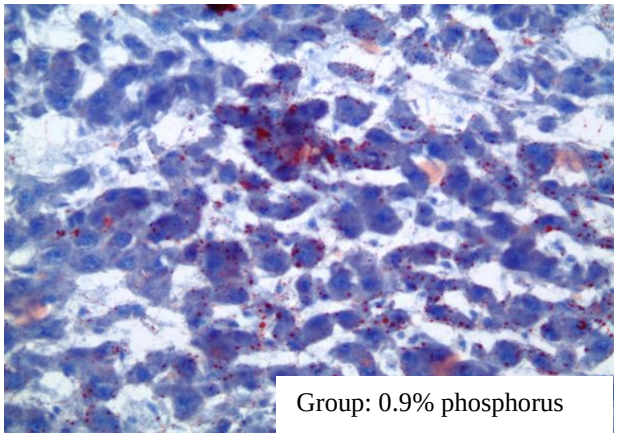




**Figure 10**: Representative images of ORO staining of liver sections from male Sprague-Dawley rats maintained for 10 weeks on high fructose diets with different level of phosphorus

# CHAPTER V

# DISCUSSION

Growing evidence suggests that excessive fructose consumption may contribute to the development of NAFLD. Mechanistically, fructose may develop NAFLD by stimulating de-novo lipogenesis, insulin resistance, oxidative stress, bacterial overgrowth, and inflammation (Longato, 2013). In an attempt to explain the association between NAFLD and fructose, a proposed mechanism is linked to hepatic ATP depletion which causes imbalance in mitochondrial homeostasis. This hypothesis was confirmed by a pilot study published in 1999 it showed an impaired ATP homeostasis in patients with NAFLD and a low recovery rate from ATP depletion as BMI increased after a bolus of fructose (Pinto et al, 1999). Similarly, Abdelmalek et al. (2012) showed that high fructose consumption in obese with type 2 diabetes had significant low ATP levels and low recovery rate from ATP depletion. The above outcomes led to the assumption that fatty liver disease, induced by high fructose intake, might be attributed to altered ATP homeostasis and can lead to low recovery from ATP depletion when individuals become overweight.

To our knowledge, no previous study examined the role of phosphorus in hepatic ATP depletion which subsequently results in fatty liver. The object of our study was to determine if there is any effect of phosphorus on the progression of NAFLD.

The effect of phosphorus on body weight and food intake has not been consistent in the literature. Our results showed that HF/0.3%P group had the lowest food intake. Phosphorus has been shown to affect food intake via several mechanisms. The production of ATP, including hepatic ATP, depends upon adequate sources of phosphorus. Furthermore, there is a lack of an adaptive mechanism that improves phosphorus absorption at low intake in a similar manner to what occurs with other micronutrients (Obeid, 2013). Therefore, phosphorus availability in food becomes an important factor that governs phosphorus levels in the circulation and its availability for ATP production. Thus the combination of high fructose-low phosphorus intake would be expected to reduce ATP production which is believed to affect food intake and energy expenditure (Obeid, 2013). Literature data indicates that increasing ATP in hepatocytes leads to a decrease in eating behavior. In addition, Koch et al showed that eating behavior is somehow related to hepatic energy metabolism, specifically hepatic ATP content (Koch et al., 1998). To support this theory, a fructose analog, the 2, 5-anhydro-D-mannitol (2,5-AM), was administrated to rats. This analogue declines the amount of inorganic phosphate necessary for ATP synthesis in the liver (Riquelme et al., 1984).This decline in liver ATP is thought to generate a neural signal that is transmitted from the liver to the central nervous system by vagal afferent neurons and concomitantly, increases food intake (Friedman et al., 2002). This observation matches only part of our results and challenges the rest since the HF/HP also showed an increase in food intake. Some studies hypothesized that other features of liver energy status which is the phosphorylation potential ([ATP]/[ADP] ratio) might be more directly

involved in the production of hepatic signal for feeding (Ji et al, 2002). Hence in the HF/ 0.15%P increased food intake is through trapping Pi and decreasing ATP production as reflected by lower ATP concentration, ATP: ADP ratio and lower phosphorylation index (Friedman, 2007). A study done by Mattar et al (2008) did not show a significant decrease in ATP content among the groups that received different percentage of phosphorus. However it rather showed an increase in glycogenesis rates with increasing dietary phosphorus which led to a decline in ATP/ADP ratio. Therefore, this drop in the ratio of ATP to ADP (phosphorylation potential) as the phosphorus increases can explain the more significant intake level for the high phosphorus group observed in our results.

On the other hand, some studies revealed that phosphorus has no effect on food intake. For example, Koshihara et al (2005) showed no significant change in food intake and weight gain in female rats that were maintained on a P-enriched diet for 42 days (Koshihara et al, 2005). Similarly, male Wistar rats food intake and weight gain maintained on a high P (1.5%) diet for 7 days did not show any alteration in food intake and body weight(Katsumata et al 2004). From these data we can conclude that there is inconsistency in the effect of phosphorus on food intake and this could be associated to numerous factors such as sex, diet component and duration of the experiment.

As for plasma phosphorus concentration, the results did not show any significant differences among the groups. This discrepancy may be due to the timing of blood sampling, and the fact that the fraction excretion of phosphorus increased with phosphorus intake. Rats were sacrificed after 14hrs fast during which there was no further ingestion of

phosphorus. Thus during this period, blood phosphate was being actively excreted which could lead to a similar blood phosphate level among the groups. Tani et al, showed a negative balance in rats given a 1.2% and 1.5% phosphorus diet. This observation could support the idea that the ingestion of high phosphorus diet could enhance the phosphorus excretion (Tani et al,2007). In addition, in our results the kidney weights/ 100g body weight was significantly higher in the high phosphorus group (0.9%P) compared to the other groups which could also strengthen the idea that renal phosphate excretion increased with high phosphorus intake.

The main purpose of this study was to examine the effect of phosphorus on the progression of non-alcoholic fatty liver disease. In the present study, six-week old male Sprague-Dawley rats fed 60% fructose were used as a nutritional model of NAFLD. NAFLD arises in association with one or more features of the metabolic syndrome specifically insulin resistance, glucose intolerance or diabetes, central obesity, dyslipidemia and hypertension (Dowman et al , 2011). There is an expansive deviation in the literature data regarding the characterization of fatty liver disorders in rats fed high fructose diet. These variances are associated with the differences among experimental protocols.

In contrast to expected outcomes based on previous studies such as Ackerman et al (2005) who reported that feeding Sprague-Dawley rats with high fructose diet (60%) for 5 weeks reproduced hepatic macrovesicular and microvesicular fat deposits, with an increase in hepatic triglycerides and hepatic cholesterol ( Ackerman et al , 2005). This study showed that Feeding Sprague-Dawley strains with high fructose diet for 10 weeks did not

significantly induce NAFLD. Several explanations may account for the inability of high fructose diets to induce fatty liver in this experiment.

It appears that individual rat strains fed a high fructose diet have different propensities to develop features analogues to NAFLD. This work has been conducted with Sprague- Dawley rats, which is an outbred strain and its genetic variability is great among colonies throughout the world. Thus, it is possible that the rats used here were resistant to diet induced fatty liver and represent a non-responsive colony of rats. Fariba et al (2013) working in Sprague-Dawley rats fed high fructose diets did not report any difference regarding histological findings in tests and control groups ( Fariba et al 2013). Likewise, stark reported adaptation of the Sprague-Dawley rats to high fat diet and high fructose diet (Stark et al 2000).

The duration of dietary treatment may also be important. In one study Kawasaki et al(2009) succeeded in developing NAFLD in fructose fed rats (60%) during a 5 week experiment (Kawasaki et al, 2009). In another study done by Spruss et al , mice provided with 30%fructose in water developed steatosis in 8 weeks ( Spruss et al, 2009). Hence, it is possible that if the experiment had been terminated earlier results would be different than those found in chronic feeding for 10 weeks.

In addition, rats' age could interfere with the development of the fatty liver model. The results of De Castro et al study revealed that adult rats (12 weeks old) fed high fructose diet showed several types of liver abnormalities, high percentages of microvesicular and macrovesicular NAFLD, and high serum levels of TG and total cholesterol. While, the

young rats (4 weeks old) showed no change in fasting glucose, total cholesterol, TG, and liver histology ( De Castro et al, 2013). Similarly, another study by De Moura et al.(2009) , noted that a high fructose diet induces metabolic syndrome more successfully in adult than in young rats ( De Moura et al,2009). These results together confirm that the disturbances that high fructose diet triggered to the liver were most evident in adult rats. Therefore, these findings can explain to some extent the absence of NAFLD in the young rats of this study (6 weeks old).

High fructose diets have been associated with weight gain in animals ( kanarek et al, 1982). The reasons for the failure to find increased body weight in the present study are unclear. One theory of the antagonist effect of fructose found in this study could be related to the method of fructose administration. In the literature, fructose was added to drinking water to develop an obesity model in rats. However adding the same amount of fructose in solid form in the diet does not produce the same response (Bray *et al.*, 2004). Mattes reported that when humans ingest energy containing beverages, energy compensation is less precise than when solid foods are ingested (Mattes et al, 1996). This is also supported by various studies which reported that providing rats with fructose in the solid form did not produce any significant increase in body weight ( Kawazaki et al (2009) and Ackerman et al (2005)). On the other hand, consumption of water enriched with 30% fructose for 8 weeks led to a significant increase in body weight (spruss et al,2009).

The results of DiMeglio and Mattes's study (2000) suggest that the subjects were able to adjust their overall daily calories intake when they consumed calories from solid foods but

not from liquid calories. In their discussion, they offered several explanations that may account for this phenomenon. First, the physical act of masticating the solid food may elicit an internal satiety signal that is not triggered by swallowing liquid calories. Also they suggested that, compared to liquids, solid foods release more of the satiety-promoting peptides, such as CCK. Besides, the differences in the volume, energy density, and osmotic properties of solids and liquids could also be involved; liquid foods empty from the stomach quicker than solid foods, thereby possibly making one feel hungrier sooner (DiMeglio and Mattes, 2000). As we know, one of the main etiological factors of liver injury is obesity and weight gain. In our present study, fructose did not lead to weight gain which could be a reason to the failure of developing NAFLD.

According to the literature, long term fructose administration in experimental animals could develop insulin resistance through the activation of JNK, changes in the gut microflora and intestinal permeability which in turn activates inflammatory pathways and through the increase in uric acid production. However, the results of this study did not show hyperinsulinemia in rats fed high fructose diet as has been seen in Hwang's study (1987). This failure in the development of insulin resistance could be a reason behind the inability of rats to significantly develop fatty liver since insulin resistance is one of the main contributors to the first hit in NAFLD pathogenesis

In this study phosphorus manipulation was shown to have effect on microvesicular steatosis. Hepatic steatosis is classified as macro or microvesicular steatosis depending on the size of the lipid vacuoles. In microvesicular steatosis hepatocytes are filled up with

numerous small lipid vesicles, which leave the nucleus in the center of the cell. While in macrovesicular steatosis, the hepatocyte contains a single, large vacuole of fat which fills up the hepatocyte and displaces the nucleus to the periphery of the cell. Whatever the cause, the presence of microvesicular steatosis is associated with the impairment of the mitochondrial β-oxidation of fatty acids (Fromenty et al, 1997). In this study the presence of microvesicular steatosis was significantly associated with phosphorus intake. A study done by Shuto et al, reported that the increase of extracellular phosphorus level can induce "ROS" production in endothelial cells. In these cells, ROS is produced mainly by the activation of NAD phosphate oxidase, xanthine oxidase and mitochondrial respiratory chain (Shuto et al, 2009). Many investigators studying hepatic injury have focused on the role of "ROS". Results from clinical trials using antioxidants in patients with NAFLD support the theory that ROS can cause steatosis as well as the hepatic injury associated with fatty liver disease (Tsedensodnom, and Sadler, 2013). The main concept connecting mitochondrial disease and ROS is that the increase in ROS production and the decrease in ATP production can cause oxidative phosphorylation deficiency, leading to mitochondrial disease.(Kirkinezoz and Moraes, 2001) As stated before, the high fructose intake in this study could lead to an increase in ROS production and a decrease in ATP availability. In addition, according to Shuto et al (2009), an increase in phosphorus intake could induce ROS formation. Hence the increase in ROS production and decrease in ATP availability could lead to a defect in oxidative phosphorylation system. When the oxidative phosphorylation system is severely impaired the $NAD^+$/NADH ratio is markedly reduced

and β-oxidation is inhibited. Impairment of β-oxidation will increase the esterification of poorly oxidized fatty acids into triglycerides, the main lipid form of that accumulates in microvesicular steatosis. One of the reasons why in this study microvesicular steatosis did not significantly progress to develop NAFLD model could be related to the ability of in particularly young rats to increase the production of UCP2 after fructose administration ( Kučera et al.,2011). UCP2 is a protein located in the inner mitochondrial membrane. Castro et al (2011) recorded an elevation in UCP2 gene and protein expression in liver homogenate of rats fed a high fructose diet. The up regulation of UCP2 is thought to act as a protective mechanism against elevated mitochondrial formation of ROS (Castro et al 2011) which leads to a decrease in the accumulation of intrahepatic lipid.

Deposition of TG in NAFLD is often found to be distributed in a zonal manner. Unfortunately not many studies report on zonal distribution of TG deposition. In humans, steatosis associated with NAFLD is described to initiate in pericentral areas with advancement to intermediate and periportal areas upon disease progression.

Normally, the periportal zone shows a higher rate of fatty acid oxidation while lipogenesis is more pronounced in pericentral zone. In this study, zone 3 distribution of steatosis (pericentral area) was the most common pattern in HF/0.15%P group (high fructose diet without phosphorus supplementation).while the zonal distribution of steatosis shifted to periportal area (zone 1) with phosphorus supplementation. Burns et al (2000) showed that in rats the fate of fructose uptake by the periportal region is either glycogen formation or oxidation. Hence, the association of fat accumulation in the periportal region with

phosphorus supplementation could be related, as stated earlier, to the fact that an increase in phosphorus will possibly increase ROS production which causes a defect in β oxidation.

In conclusion, these results indicate that healthy young animals were able to adapt to the high fructose diet without developing metabolic disorders.

Young male Sprague - Dawley rats fed a high fructose diet (60%) for 10 weeks may not be a suitable model for investigating the effect of phosphorus on the progression of NAFLD.

# CHAPTER VI

# CONCLUSION AND RECOMMENDATIONS

This study was an attempt to investigate whether phosphorus is a factor in the development of NAFLD. It was hypothesized that a high fructose diet induces NAFLD through sequestering phosphorus and making it unavailable for the production of ATP. Previous studies have shown that fructose enriched diets fed to experimental animals are good models of NAFLD.

In our study, high fructose diet did not have any effect on the liver of our rats. Keeping in mind that Sprague-Dawley rats are an out-bred strain where different colonies over the world can possess great genetic variability. Potential explanations for the discrepancies found between our results and those reported in literature could be related to several factors such as:1- the variability in the composition of diets; 2- different forms of fructose administration, such as in the diet or water (Joyeux et al,2006, Sanchez et al,2007); 3-duration of dietary treatments, from weeks to months(Sanchez et al,2007,Lee et al,2006); 4- use of different rodent models (Lee et al,2006); and different age of animals, i.e., young (Barbosa et al,2007, Joyeux et al,2006) or adults (Sanchez et al,2007).

It is recommended to select the animal model that best answers the research question. The majority of animal models focus on providing a diet that causes liver damage. Along with high fructose diet, the model used most often is the MCD diet, a diet high in

sucrose and fat but low in methionine and choline which are essential in hepatic B-oxidation and VLDL synthesis. Another dietary model is made up of giving an atherogenic diet, high in cholesterol and butter fat; yet, this model did not mimic the human NASH metabolic manifestations. (Hebbard, and Jacob 2011) Several models used a high-fat diet using more than 70% of energy from fat; although the high fat diet yielded pathological manifestations that resemble human NASH, the results also depend on the type of fat, the fat content, the rodent species and strain.

Based on our results, it is probably more advisable that future studies use adult rats in the fructose enriched diet model. Furthermore, providing fructose in drinking water rather than in solid food could have more influence on liver and body weight.

The mechanism underlying fructose induced fatty liver disease remains poorly understood and might be related to the phosphorus concentration inside the hepatocytes. This hypothesis, if proven true, could be the basis of future research and could possess a public health implication.

# APPENDIX

**Appendix 1**

The mineral mix used is the AIN-93G mixture purchased from Dyets inc.

Experimental diets & Ingredients for Laboratory Animals used at 35 g/kg diet.

The mixture is composed of the following:

| Ingredients | g/kg mixture |
|---|---|
| Calcium Carbonate | 357 |
| Potassium Phosphate (monobasic) | 196 |
| Potassium citrate $H_2O$ | 70.78 |
| Sodium Chloride | 74 |
| Potassium Sulphate | 46.6 |
| Magnesium Oxide | 24 |
| Ferric Citrate, U.S.P | 6.06 |
| Zinc Carbonate | 1.65 |
| Manganous Carbonate | 0.63 |
| Cupric Carbonate | 0.3 |
| Potassium Iodate | 0.01 |
| Sodium Selenate | 0.01025 |
| Ammonium Paramolybdate 4 $H_2O$ | 0.00795 |
| Sodium Metasilicate 9 $H_2O$ | 1.45 |
| Chromium Potassium Sulfate 12 $H_2O$ | 0.275 |

**Appendix 2**

Phosphorus free mineral mix used is Phosphorus Deficient AIN-76 Salt Mix (use at 35 g/Kg diet) from Dyets. Inc. Experimental Diets & Ingredients for Laboratory Animals.

The mixture is composed of the following:

| Ingredients | g/kg mixture |
|---|---|
| Calcium Carbonate | 368 |
| Sodium Chloride | 74 |
| Potassium Citrate, monohydrate | 220 |
| Potassium Sulfate | 52 |
| Magnesium Oxide | 24 |
| Manganous Carbonate | 3.5 |
| Ferric Citrate | 6 |
| Zinc Carbonate | 1.6 |
| Cupric Carbonate | 0.3 |
| Potassium Iodate | 0.01 |
| Sodium Selenite | 0.01 |
| Chromium Potassium Sulfate | 0.55 |
| Sucrose, finely powdered | 250 |

**Appendix 3**

The vitamin mix used is the AIN-76A mixture purchased from Dyets inc.

Experimental diets & Ingredients for Laboratory Animals.

The mixture is composed of the following:

| Vitamin | g/kg mixture |
|---|---|
| Thiamin HCl | 0.60 |
| Riboflavin | 0.60 |
| Pyridoxine HCl | 0.70 |
| Niacin | 3.00 |
| Calcium Pantothenate | 1.60 |
| Folic acid | 0.20 |
| Biotin | 0.02 |
| Vitamin $B_{12}$ (0.1%) | 1.00 |
| Vitamin A palmitate (500,000 IU/g) | 0.80 |
| Vitamin $D_3$ (400,000 IU/g) | 0.25 |
| Vitamin E acetate (500 IU/g) | 10.00 |
| Menadione Sodium Bisulfite | 0.08 |
| Sucrose finely powdered | 981.1 |

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