AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF PHOSPHORUS SUPPLEMENTATION ON THE DEVELOPMENT OF FATTY LIVER INDUCED BY A LOW PROTEIN DIET IN RATS

by
ROLA USAMA HAMMOUD

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submitted in partial fulfillment of the requirements
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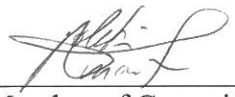

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AN ABSTRACT OF THE THESIS OF

Rola Usama Hammoud for Master of Science
Major: Nutrition

Title: The Effect of Phosphorus Supplementation on the Development of Fatty Liver Induced by a Low Protein Diet in Rats

There has been an increasing bulk of literature addressing the effect of varying levels of dietary protein intake on weight changes, body composition, and disease risk. More recent studies showed the epigenetic effect of a protein-restricted diet on the long-term development of the metabolic syndrome. Inadequate dietary protein intake is also known to be associated with the development of protein energy malnutrition (PEM). Kwashiorkor is a major form of PEM that is distinctively associated with edema and nonalcoholic fatty liver disease (NAFLD). However, its etiology remains unclear. Epidemiological evidence has reported that Kwashiorkor is mostly prevalent in developing countries where the main staple foods include plant-based food items such as maize, rice, or cassava, which are known to be poor dietary sources of both protein and phosphorus. Furthermore, malnutrition is commonly associated with low total body phosphate. Therefore, inadequate dietary P intake may be an important factor in the etiology of Kwashiorkor. However, the independent effect of P on the outcomes associated with a low protein diet remains unclear.

The purpose of this study is to dissect the exclusive role of varying concentrations of dietary P on weight gain, food intake, body composition, serum lipid profile, serum glucose, serum insulin levels, and liver fat content in rats maintained on a low protein diet for 9 weeks.

Forty-nine Sprague-Dawley rats were randomly allocated to 5 groups and fed ad-libitum 5 iso-caloric diets that only varied in protein and P concentrations. The control group received a normal protein (20%) from egg white, and normal P (0.3%) diet, whereas the 4 other groups were maintained on low protein (10%) diets with varying levels of P: 0.015%, 0.056%, 0.1%, & 0.3%. The rat weights and food intakes were measured twice per week for 9 weeks. The rats were then sacrificed, and their body composition, liver fat content, and plasma biomarkers were analyzed.

The results showed the average food intake, weight gain, and final body weight per group over the 9 week period were the highest in the control group, whereas the lowest food intake and weight gain was observed in the 0.015% P group. Food intake and weight gain improved with the increase in P level among the low protein groups. Additionally, body composition in the control, and 0.3% P groups had lower percentage of water when compared to 0.015% group. Hence, adequate dietary P may be decreasing the risk of developing symptoms of Kwashiorkor while maintained on a low protein diet. However, maintaining Sprague-Dawley strains on a low protein diet for 9 weeks did not induce NAFLD, so the effect of phosphorus on the progression of the disease could not be assessed.

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ABBREVIATIONS

%	Per Cent
&	And
/	Per
<	Less Than
=	Equal To
>	Greater Than
±	Plus or Minus
X	Multiply
^{0}C	Degrees Celsius
AI	Adequate Intake
ATP	Adenosine Triphosphate
AUB	American University of Beirut
BP	Boiling Point
CO2	Carbon Dioxide
DRI	Dietary Reference Intake
ECF	Extracellular Fluid
EDTA	Ethylenediaminetetraacetic acid
EE	Energy Efficiency
FFA	Free fatty acids
FI	Food Intake
G	Gram
H&E	Hematoxylin And Eosin
HDL	High Density Lipoprotein
IACUC	Institutional Animal Care And Use Committee
Kcal	Kilocalorie
Kg	Kilogram
KH2PO4	Monopotassium phosphate
Kj	Kilo Joule
LDL	Low Density Lipoprotein
Mg	Milligram
mL	Milliliter
MW	Molecular Weight
N	Number
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic Steatohepatitis
ORO	Oil-Red-O
P	P-value
P	Phosphorus
PEM	Protein Energy Malnutrition

PO_4	Phosphate
RDA	Recommended Dietary Allowance
SD	Standard Deviation
SREBP1-c	Sterol Regulatory Element Binding Protein-1c
STAT 3	Signal Transducer and Activator of Transcription 3
T2DM	Type-2 Diabetes Mellitus
TG	Triglyceride
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
Wt	Weight

CHAPTER I

LITERATURE REVIEW

A. Protein

1. Overview

Protein serves both a structural and functional role in the body. Upon digestion and absorption, dietary proteins are broken into amino acids, which serve as the primary units in the synthesis of enzymes, cell membranes, hormones, transport proteins, and many other components that are indispensable for the body's normal metabolic function, growth and overall health.

According to the WHO report on protein and amino acid requirements in human nutrition (WHO, 2007), adequate dietary protein requirements are associated with the energy needs and the overall macronutrient and micronutrient composition of the diet. This is due to the complex interactions between different nutrients, and the integration between different metabolic pathways. For instance, the pathways of amino acid metabolism are influenced by micronutrient status, where an inadequate mineral or vitamin status may lead to disturbances in the amino acid metabolic pathways. Generally, amino acids and body protein homeostasis is regulated through major metabolic systems. These include protein synthesis, protein degradation, amino acid oxidation, and amino acid *de novo* synthesis in the case of some nonessential amino acids. (Figure1) The rate of activation of each system will affect the nitrogen balance, and its equilibrium is highly dependent on energy, and dietary protein intake (Young & Marchini, 1990).

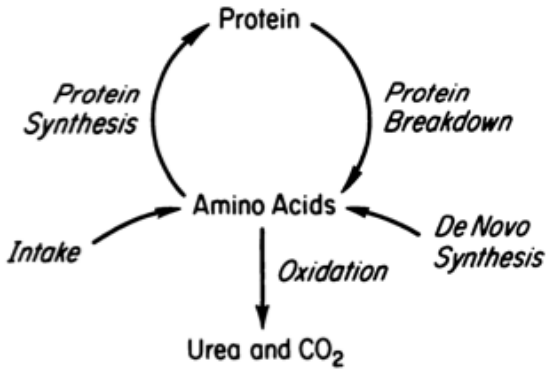

Figure 1: General Amino Acid Metabolism (Young & Marchini, 1990)

The recommendation for healthy adults to reduce the risk of chronic diseases is to divide macronutrient intake into around 30% of total energy from fat, 15 % from protein, and 55-60% from complex carbohydrates (Krauss et al., 2000). The dietary reference intakes (DRIs) established for dietary protein are shown in (Table 1).

Table 1 Dietary Reference Intakes (DRIs) for protein in g/kg/day according to age group

Age Group	**DRI (g/kg/day)**
Infants	1.5
1-3 years	1.1
4 – 13 years	0.95
14 – 18 years	0.85
Healthy adults	0.8
Pregnant and lactating women	1.1

The most important dietary sources of protein come from animal products, such as meat, fish poultry, eggs, and dairy products. These sources serve to provide all the essential amino acids, unlike other sources of "incomplete" proteins, which include legumes, grains, nuts, seeds, and vegetables (DRI, 2002/2005).

2. Health Impacts

There has been an increasing bulk of literature addressing the effect of higher dietary protein intake on weight changes, body composition, and disease risk. Evidence has shown that high protein diets decrease the risk of developing metabolic syndrome and cardiovascular disease by improving blood lipid profiles, and glycemic control (Wolfe & Piche, 1999; Seino et al., 1983; Piatti et al., 1994). A study comparing the effects of two energy restricted diets that only differed in their protein to carbohydrate ratio showed that the higher protein diet (23%) significantly reduced blood triglyceride levels as opposed to the low protein diet (10%). It also improved body composition by preserving lean body mass and had a lower glycemic response (Farnsworth et al., 2003). Another study assessed the long-term effect of an increased protein to carbohydrate ratio in the diet on weight changes, blood lipid profile, and body composition. The high protein diet (1.6g/kg/day) had a more favorable effect on the blood lipid profile by reducing the triglyceride (TG) level, and raising HDL cholesterol levels as compared to the lower protein diet (0.8g/kg/day). The high protein diet also improved body composition by aiding in fat mass loss (Layman et al., 2009).

On the other hand, a low protein diet has been found to be associated with various unfavorable health effects. More recent literature has addressed the epigenetic effect of a protein-restricted diet and its long-term effect on the development of the

metabolic syndrome. The metabolic syndrome is strongly correlated with high blood cholesterol, insulin resistance, hypertension, and increased central obesity (Mathieu et al., 2006). A recent study examined the epigenetic effect of a protein-restricted diet in lactating rats on the development of steatohepatitis, which is a feature of the metabolic syndrome. The results showed that a protein-restricted diet during lactation induced steatohepatitis and oxidative stress via the activation of the leptin/STAT3 signaling pathway (Kwon et al., 2012). More evidence showed that maternal protein restriction might lead to disordered lipid metabolism, and the long-term development of metabolic syndrome in the offspring (Erhuma et al., 2007).

B. Protein Energy Malnutrition:

Inadequate dietary protein intake is known to be associated with several adverse health effects including the development of protein energy malnutrition (PEM). Severe malnutrition, including PEM, poses a serious public health problem, since it has been reported to contribute to up to 50% of childhood mortality in developing countries (Kimutai et al., 2009). Findings from the World Health Organization (WHO) have documented that more than a third of the world's children are affected by PEM, where 80% are distributed in Asia, 15% in Africa, and 5% in Latin America (De Onis et al., 1993). Malnutrition is generally defined as the condition arising from the inadequate intake of macro and/or micronutrients. In many cases inadequate protein and energy intake can lead to what is known as protein energy malnutrition. PEM is a term that defines a group of disorders and these include Kwashiorkor, Marasmus, and intermediate states of Kwashiorkor-marasmus (Batool et al., 2015).

The word Kwashiorkor originated from Ghana, and it is an African term referring to young children between the ages of 4 months to 5 years who have been weaned from breast-feeding. This syndrome was described as a result of a lack of protein intake combined with an excess of carbohydrates (Castiglia, 1996). Kwashiorkor is the edematous form of protein-energy malnutrition and what makes it distinctive from marasmus is the presence of edema, hypoalbuminemia, and dermatosis (Liu et al., 2001). Epidemiological evidence shows that the prevalence of edematous malnutrition such as Kwashiorkor is correlated to populations whose staple food is low in protein such as cassava, or contains a poor quality of protein such as maize (Waterlow, 1997). According to Muller & Krawinkel, (2005) poverty is the main contributing factor of malnutrition.

The pathophysiology of Kwashiorkor is associated with cell membrane damage and multiple organ dysfunctions. It is also coupled with reduced whole body potassium, magnesium and phosphorus, which in severe cases may lead to hypokalemia, hypomagnesaemia, and hypophosphatemia. Furthermore, renal function and cardiac output have been both reported to be compromised in Kwashiorkor (Manary et al., 2009). Kwashiorkor is also associated with hypoprotenemia, a common feature of PEM, and altered body fluid compartments specifically a relative increase in extracellular fluid (ECF), which is apparent in children suffering from edema (Oshikoya & Senbanjo, 2009). Additionally, a prominent feature of Kwashiorkor that differentiates it from marasmus is the accumulation of lipids in the hepatocytes and the development of fatty liver (Bandsma et al., 2010). Fatty liver has been reported to be associated with edema in PEM (Waterlow, 1997).

Low total body phosphorus is commonly observed in cases of protein energy malnutrition. PEM is commonly observed in populations whose staple foods are mainly maize, cassava, or rice (Manary et al., 2009). These dietary components are known to be poor dietary sources of both protein and phosphorus. Furthermore, populations that are at high risk of developing Kwashiorkor consume minimal amounts of animal and dairy products, which are known to be the most important dietary sources of protein. Likewise, animal and dairy products are also important sources of phosphorus. Plant-based foods such as legumes, and cereals contain the majority of their P in the form of phytate phosphorus, which has a low bioavailability for absorption. Therefore, we can speculate that inadequate dietary P intake may also be a factor in the development of PEM. However, the sole involvement of P in the outcomes associated with low protein diets remains unclear.

C. Phosphorus

1. Overview

Phosphorus is a multivalent non-metal element that is rarely found in nature in its free form; hence it is mostly present in the anion form PO_4 (Kalantar et al., 2010). It is an essential mineral in the human body, and it plays an important role in cellular metabolism, since it is a major constituent of key enzymes involved in phosphorylation, and is also integrated with adenosine triphosphate (ATP). It also functions as a urinary buffer by maintaining acid-base homeostasis (Amanzadeh & Reilly, 2006). Additionally, phosphorus is involved in bone development, and normal functioning of blood cells, muscles, and the nervous system (Kimutai et al., 2009; Freiman et al., 1982). On average, the total body phosphorus content is 700g of which 85% is found in

bones and teeth, 14% is in soft tissues, and 1% is in the extracellular fluid. The average dietary intake of phosphorus is approximately 800-1400 mg/day, and the normal serum phosphorus concentration should be between 2.5-4.5 mg/dl or 0.8 – 1.45 mm/l (Amanzadeh & Reilly, 2006). According to the Food and Nutrition Board of the Institute of Medicine, the recommended daily allowance for phosphorus for both male and female adults over the age of 19 years is 700 mg/day. (See Table 2)

Table 2: Recommended Dietary Allowance (RDA) in mg/day for P by Age Group (National Academy of Science, 1997)

Age Group	RDA (mg/day)
1 through 3 years	460
4 through 8 years	500
9 through 18 years	1250
19 through 70 years	700
>70 years	700

Phosphorus is naturally present in foods that are rich in protein. Protein rich foods, such as animal products, contain organic phosphorus, which is hydrolyzed in the intestine and absorbed in its inorganic form PO_4. Generally, around 40 to 60% of dietary phosphorus is absorbed in the small intestine. As opposed to organic phosphorus, inorganic phosphorus in the form of salts can be found in supplements, medications, and foods containing P additives, and it is >90% absorbed by the intestine, since it readily occurs in a free and unbound form (Kalantar et al., 2010). Phosphorus

absorption mainly occurs at the level of the jejunum and ileum. The mode of absorption is primarily through concentration-dependent passive transport, whereas a smaller portion is absorbed by active transport, which is facilitated by 1,25-dihydroxyvitamin D (Kalantar et al., 2010; Davis et al., 1983).

Animal-based foods such as dairy products, meat, poultry, and fish have a higher digestibility of P than plant proteins. Table 3 shows the various animal and plant dietary sources of phosphorus (Kalantar et al., 2010). There is a known strong and direct correlation between dietary protein intake and phosphorus intake. A study done on patients with chronic kidney disease used a food frequency questionnaire to develop a regression equation that explains the relationship between protein and P intake: Dietary P (mg) = 128 mg P + (dietary protein in g) x 14 mg P/g protein (Boaz et al., 1996).

Plant-based foods, such as cereals, and legumes, are the main staple foods in many developing countries. Unrefined whole grain cereals such as whole wheat, or brown rice contain high amounts of P however it is mainly bound in the form of phytate, which in turn renders it unavailable for absorption. (Table 4)

Table 3: Dietary P and protein content and P-to-protein ratio in selected food items (Kalantar et. al 2010)

Food Item	P (mg/100g)	Protein (g/100g)	P-to-Protein ratio (mg/g)
Animal-based food			
Egg White	12.5	9	1.4
Lamb	200	32	6.3
Tuna (canned in water)	164	25.5	6.4
Chicken Breast	231	31	7.5
Ground Beef	194	25.8	7.5
Whole Egg	140	10.5	13.3
Dairy Products			
Mozzarella cheese	526	26	20.1
Milk, low fat (2%)	93.5	3.3	28.3
Cottage Cheese	131	12.2	10.7
Plant-based food			
Soy beans	211	14.3	14.7
Peanuts	356	23.6	15.1
Edamame	161	10.3	15.6
Black beans	140	9	15.8
Lentils	178	8.9	20.0

Table 4 Total and phytate phosphorus content of selected plant-based food items (Ravindran et al., 1994)

Food Item	Phosphorus (g/100g)		Phytate-P % of Total
	Total	**Phytate-P**	
Maize	0.26	0.22	84.6
Rice Brown (unpolished)	0.38	0.28	73.7
Rice (polished)	0.31	0.17	54.8
Wheat Bran	1.15	1.03	76.9
Cassava	0.16	0.04	25
Sweet Potato	0.21	0.05	23.8
Potato	0.24	0.05	20.8
Soy Bean	0.6	0.37	61.7
Lentils	0.31	0.2	64.5

Phytate is the principal storage form of phosphorus in plants. It is formed during maturation of the seed and it represents 60-90% of the total phosphate. Humans and monogastric animals cannot hydrolyze and absorb phytate since they lack the necessary enzyme phytase (Kumar et al. 2010). On the other hand, refined cereals, and cereal products that are subjected to multiple forms of processing such as milling, extraction, and fermentation contain less phytate. To illustrate, polished rice contains approximately 20% less phytate than unpolished brown rice (Table 4). However, it is known that processing and refining cereals also leads to around 70% reduction in essential minerals, such as potassium, phosphorus, and magnesium (Obeid et al., 2014).

Therefore, whole grains, refined cereals, and plant-based food products are relatively inadequate sources of phosphorus when compared to animal-based food.

2. Health Impacts

To this day, not many studies have been done to obtain a greater understanding on the possible association between dietary phosphorus intake and fatty liver development. However plenty of evidence has shown an association with phosphorus and glycemic control and the lipid profile (Obeid et al., 2014). For example, several studies have reported that the increase in serum phosphate and in phosphorus intake of non-diabetic subjects was associated with an improvement in glucose tolerance and insulin sensitivity (Obeid, 2013). The possible mechanism to explain these results could be attributed to Adenosine triphosphate (ATP) production, which is dependent on phosphorus availability (Solomon & Kirby, 1990; Morris et al., 1978). ATP is necessary for multiple metabolic processes. Since ATP acts as a phosphate donor for many metabolic reactions, it is postulated that insulin release stimulates the phosphorylation of many compounds; this may compromise phosphorus availability for ATP production. Therefore, increased insulin release under low phosphorus diet exacerbates the situation. Moreover, reduced phosphorus peripheral uptake due to impaired glucose tolerance was also suggested to affect thermogenesis (Obeid, 2013). A previous study has found that the inclusion of phosphorus in oral glucose load was able to improve insulin sensitivity (Khattab et al., 2011), and this may probably relate to its capacity to trap glucose within the cells as a result of its phosphorylation (Obeid et al., 2014).

Furthermore, a study examined the effect of high amounts of dietary phosphorus in gelatinized potato on lipid metabolism of rats. The results showed rats on higher phosphorus diet had a lower hepatic triglyceride level. However this lipid lowering effect could be attributed to the slowly digestible starches (Kanazawa et al., 2007). Another study investigated the effect of dietary P consumption on cholesterol metabolism in mice. The results showed that dietary phosphorous restriction considerably increased hepatic lipid-accumulation induced by a high-cholesterol diet (Tanaka et al., 2013). In another study, of adult male Sprague-Dawley rats examined the effect of phosphorus on glycogenesis and lipogenesis when rats were fed a high fructose diet. The rats were kept on a high-fructose diet for 4 days with different P content (0.15%, 0.165%, 0.30%, and 1.65%). In vivo rates of glycogen and lipid synthesis and hepatic glycogen levels were measured. The results showed that increased dietary phosphorus was associated with an increase in postprandial epididymal fat pad ($p = .007$) and hepatic lipogenesis ($p = .029$), as well as glycogenesis ($p = .024$) (Mattar et al., 2010).

To our knowledge, no animal models have examined the exclusive effect of varying phosphorus concentrations on the development of fatty liver in the case of a low protein diet.

D. Nonalcoholic Fatty Liver Disease:

1. Overview

Nonalcoholic Fatty Liver Disease (NAFLD) is a term used to describe the condition in which excessive liver fat accumulation occurs independent of a history of alcohol abuse (Takahashi et al., 2012; Sass et al., 2005).

NAFLD is prevalent in approximately 10–20% of the world population, affecting both the adults and the pediatric population (Lewis & Mohanty, 2010; Sass et al., 2005). It is known to be the most common liver disease in the United States and the western world and its prevalence is reported to be persistently increasing (Cave et al., 2007; Kucera & Cervinkova, 2014). Plenty of evidence has shown a correlation in the prevalence of NAFLD in patients diagnosed with metabolic syndrome (Lewis & Mohanty, 2010).

The pathogenesis of NAFLD has been shown to be similar to that of metabolic syndrome and insulin resistance (Kucera & Cervinkova, 2014). The disease is represented in two forms: simple steatosis, and nonalcoholic steatohepatitis (NASH). NASH is a more severe form of the disease that is presented as liver fat accumulation in addition to an inflammatory response. It can progress to liver cirrhosis and possibly hepatocellular carcinoma (HCC) (Takahashi et al., 2014).

Hepatosteatosis may occur in two different types; macrovesicular and microvesicular. Macrovesicular steatosis is common in alcoholic liver disease, obesity, diabetes, and Kwashiorkor. It appears as one or a few lipid droplets filling the total hepatocyte. On the other hand microvesicular steatosis is common in mitochondrial disorders, fulminant hepatitis D, and Reye syndrome, and it occurs as multiple small lipid droplets giving a foamy appearance (Ozturk & Soylu, 2014).

The mechanism for fatty liver development is not restricted to one single metabolic pathway. Free fatty acids (FFA) are found in the liver from three different sources, lipolysis of the adipose tissue, dietary sources, and *de novo* lipogenesis. Additionally, the fate of free fatty acids in the liver is to either be utilized through β-oxidation, or re-esterified to triglycerides and stored as lipid droplets, or packaged and

exported as very low-density lipoprotein (VLDL) (Dowman et al., 2010). Therefore hepatic fat accumulation could occur due to a dysfunction in more than one metabolic pathway. It could be due to decreased fatty acid beta-oxidation, or an increase in fatty acid synthesis and delivery to the liver, or it could be due to inadequate export of triglycerides from the liver in the form of very low-density lipoprotein (VLDL) to peripheral tissues (Figure 2) (Lim et al., 2010). A common approach is used to describe the pathogenesis of NAFLD namely NASH, and it is referred to as the multiple "hit", or the "two-hit" hypothesis. In this hypothesis, fatty liver is attributed to the occurrence of multiple risk factors or events. Initially, hepatic fat accumulation could occur due to dietary, environmental or possibly genetic factors. Secondly, progressive liver damage might occur due to increased oxidative stress and increased inflammation (Wree et al., 2013; Gusdon et al., 2014).

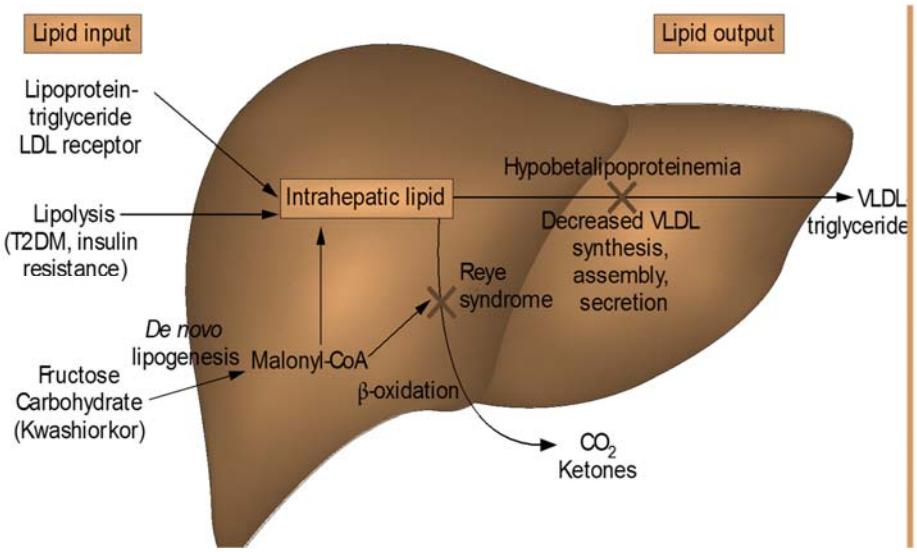

Figure 2 Pathways of hepatic lipid metabolism and possible factors that can lead to increased intrahepatic lipid accumulation (Lim et al., 2010)

2. The Role of Nutrition in NAFLD

Nutrition plays a significant role as an external risk factor for the development of NAFLD (Cave et al., 2007). Extensive research is still in progress to further understand the nutritional effect on the development of non-alcoholic fatty liver disease (Zivkovic et al., 2007). Many studies have been conducted to examine the effect of high fat as well as high carbohydrate diets on the development of NAFLD. Plenty of evidence has revealed a strong correlation between NAFLD and the metabolic syndrome (Lewis et al., 2010). Around 53% of patients in the USA, with hepatic steatosis, have also been reported to suffer from the metabolic syndrome; this prevalence increases to 88% in patients suffering from NASH (Marchesini et al., 2003).

Several studies have reported that increased saturated fatty acid intake is associated with increased insulin resistance, which can lead to the development of fatty liver (Kani et al., 2014). Additionally, some studies showed that individuals suffering from NASH consume a higher percentage of their energy from fat. However a study utilizing stable isotopes showed that only 15% of intrahepatic lipids originated from dietary fat (Donnelly et al., 2005). Therefore, although it is not the exclusive contributor, a high fat diet may be considered as an important nutritional risk factor for the development of NAFLD (McCarthy et al., 2012). Recently, researchers have displayed great interest in examining the effects of carbohydrate-sweetened beverages, sucrose and fructose, on the development of metabolic syndrome and the increased risk of NAFLD. Current data showed that sweetened beverage consumption was associated with increased liver fat accumulation, oxidative stress, and insulin resistance (Asrih & Jornayvaz, 2014; Assy et al., 2008; Abid et al., 2009). Likewise, other studies done on animal models have shown that rats fed a high-carbohydrate and low-protein diet

presented with an increase in liver lipid content (Menezes et al., 2013). However, in regards to dietary protein intake, very few clinical studies have examined its effect on the development of metabolic syndrome and NAFLD (McCarthy et al., 2012).

Nonetheless, several studies have observed the association of protein energy malnutrition (PEM), namely Kwashiorkor, with changes in insulin sensitivity, serum lipid profile, and hepatic lipid accumulation (Williams et al., 2003; Doherty et al., 1992). Although NAFLD is the most recurring chronic liver disease in wealthy communities, mainly due to a westernized diet characterized by excessive energy consumption, and the increased intake of some macronutrients such as saturated fatty acids, refined carbohydrates, and fructose (Ouyang et al., 2008; Thuy et al., 2008), hepatic fat accumulation has also been observed in cases of malnutrition, namely Kwashiorkor (Kwon et al. 2012; Doherty et al. 1992).

E. NAFLD and Kwashiorkor

Researchers have been investigating the serological and the endocrine changes that are linked with Kwashiorkor to obtain a better understanding of the etiology of fatty liver in response to PEM. Although extensive research has been done, the factors that cause the development of fatty liver in response to protein malnutrition remain unclear. Nonetheless, many hypotheses have been developed in an attempt to explain the etiology of fatty liver in cases of Kwashiorkor. However till now there is no definite answer or theory developed to explain the mechanism of this disease. One hypothesis mentions the effect of free radical accumulation on the development of fatty liver. The free radical hypothesis was proposed by Golden & Ramdath (1987), who stated that the etiology of Kwashiorkor is associated with the accumulation of free radicals in the body

due to an overall decrease in free radical scavengers, and other "protective mechanisms", as a result of malnutrition and dietary deficiency of macro and micronutrients (Figure 3). This hypothesis was supported by several studies that observed low levels of the scavenger glutathione in children suffering from Kwashiorkor (Roediger & Waterlow, 1995). This accumulation of free radicals according to Golden and Ramdath would then result in protein damage leading to the symptoms observed in Kwashiorkor such as edema, fatty liver, skin and hair pigment alterations, diarrhea, and immune dysfunction (Golden & Ramdath, 1987).

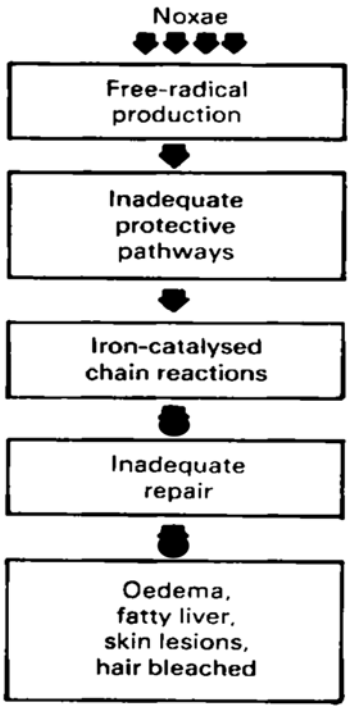

Figure 3: Proposed steps in the pathogenesis of Kwashiorkor (Golden&Ramdath, 1987)

An important feature of fatty liver was its association with glucose intolerance, and it has been reported that children with Kwashiorkor showed signs of glucose intolerance and decreased insulin sensitivity. In one study, blood samples and muscle

biopsies were examined in malnourished children, recovered children, and normal children. The results showed the anaerobic metabolism of glucose, and lactate production was impaired in malnourished and recovered children when compared with normal children (Alleyne et al., 1972). This evidence suggests that insulin resistance in Kwashiorkor may occur at the post-receptor level (Fong et al., 2000). On the other hand, a study done on 17 adults with severe protein energy malnutrition assessed the glucose tolerance and insulin response to glucose infusion. The study showed that the insulin secretory response is severely diminished in patients with PEM (Smith et al., 1975).

A mechanism that links the role of insulin resistance in the accumulation of hepatic fat and the development of fatty liver is illustrated in figure 4, where insulin plays a significant role in the suppression of adipose tissue lipolysis. Nevertheless, in situations of insulin resistance, this suppression is impaired which leads to an increase in the efflux of FFA from adipose tissue. The hyperinsulinaemia associated with IR leads to the up-regulation of the transcription factor sterol regulatory element binding protein-1c (SREBP-1c), which is a key transcription factor that regulates genes involved in denovo-lipogenesis, and inhibits beta-oxidation of FFAs thus further stimulating hepatic lipid accumulation (Dowman et al., 2010).

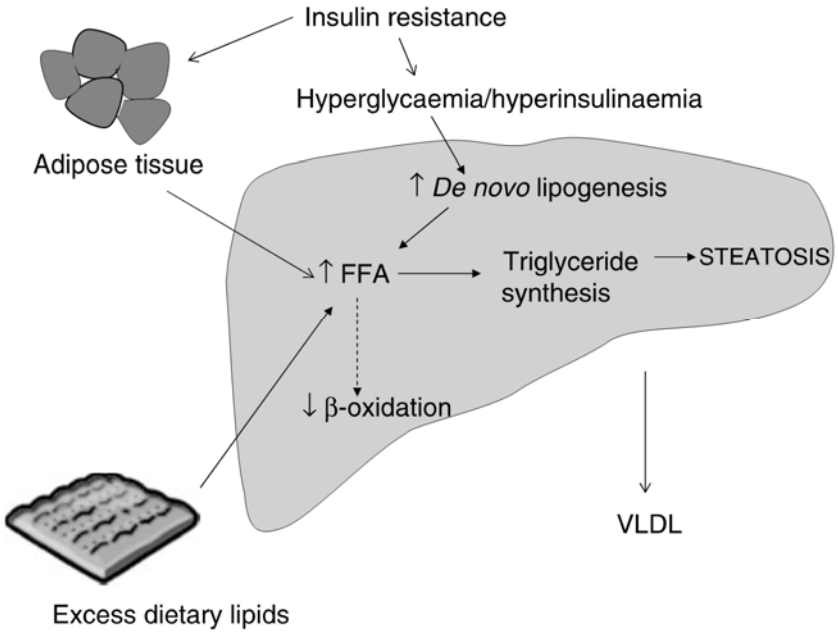

Figure 4 The role of insulin resistance in hepatic fat accumulation (Dowman et al., 2010).

In conclusion, Kwashiorkor is a form of PEM that is distinctively associated with edema and NAFLD, and it is typically associated with multiple metabolic disorders. It is also linked with electrolyte imbalance, including low total body phosphate. PEM is mostly prevalent in developing countries where the main staple foods include plant-based food items such as maize, rice, or cassava, which are known to be poor dietary sources of both protein and phosphorus. Phosphorus is the second most abundant mineral in the human body; however, its depletion is commonly observed in PEM. Therefore, inadequate dietary P intake may be an important factor in the development of PEM. However, the independent effect of P on the outcomes associated with a low protein diet remains unclear.

Therefore the purpose of this study is to dissect the exclusive role of varying concentrations of dietary phosphorus on weight gain, food intake, body composition, serum lipid profile, serum glucose, serum insulin levels, and liver fat content in 49 Sprague-Dawley rats maintained on a low protein diet for 9 weeks.

CHAPTER II

MATERIAL AND METHODS

A. Experimental Procedure

1. Animal Housing

Forty-nine six-week old male Sprague-Dawley rats weighing between 200-220 g were used. The rats were housed individually in regular cages at 22±1°C in a light/dark cycle of 12/12 hours. The animals had free access to water and were fed ad libitum on a control diet for one week to familiarize them to the environment and diet.

2. Experimental Diet

The experimental diets were all prepared using the same ingredients and the diets were iso-caloric. The dietary ingredients include corn oil, dried egg white, cornstarch, sucrose, cellulose, phosphorus free mineral mix, vitamin mix, and potassium phosphate. Dried egg white was used as the main source of protein since it is known to have negligible amounts of P but supplies all the essential amino acids (Table 5).

Table 5 Dietary Composition (macro and micronutrients) given to the 5 rat groups

Ingredients	Group 1 Control Diet	Group 2	Group 3	Group 4	Group 5
Diet g/100g	**% (Kcal)**				
Egg white (protein)	20 (80)	10 (40)	10 (40)	10 (40)	10 (40)
Corn Oil (fat)	10 (90)	10 (90)	10 (90)	10 (90)	10 (90)
Corn Starch (CHO)	30 (120)	35 (140)	35 (140)	35 (140)	35 (140)
Sucrose (CHO)	30 (120)	35 (140)	35 (140)	35 (140)	35 (140)
Cellulose	7%	7%	7%	7%	7%
Mineral mix *	2%*	2%*	2%*	2%*	2%*
Vitamin mix	1%	1%	1%	1%	1%
Total Energy (Kcal)	410	410	410	410	410
Diet g / Total Energy (Kcal)					
Egg white** Of which %Phosphorus	22.5 g 0.03%	10.25 g 0.015%	10.25 g 0.015%	10.25 g 0.015%	10.25 g 0.015%
Corn Oil	5 g	4.5 g	4.5 g	4.5 g	4.5 g
Corn Starch	30.75 g	35.875 g	35.875 g	35.875 g	35.875 g
Sucrose	30.75 g	35.875 g	35.875 g	35.875 g	35.875 g
Phosphorus (added) %	0.2822%	-	0.0411%	0.0911%	0.2911%
Total Phosphorus %	0.312%	0.015%	0.056%	0.106%	0.306%

*Phosphorous Free
** 1.5mg Phosphorus/ 1 g Egg White
MW KH_2PO_4 = 136 g of which P = 31 g

3. Experimental Design

This study received approval by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut (AUB). Following the one-week adaptation period, the rats were randomly allocated to five experimental groups. The groups were divided as follows: (Table 5)

- Group 1 (Control): 20% protein and 0.3% P
- Group 2: 10% protein and 0.015%P
- Group 3: 10%protein and 0.056%P
- Group 4: 10% protein and 0.1%P
- Group 5: 10% protein and 0.3%P

The rats were kept on their corresponding experimental diets for 9 weeks and their food intake and body weight were measured twice per week. Food intake measurement was done by subtracting the weight of the food plate on each day of weighing from the weight from the previous day of weighing, taking into account any possible food spillage.

After 9 weeks of follow up, on the final day, the rats were fasted overnight. The following morning the rats were placed under anesthesia and were dissected. Blood samples were drawn from each rat, and collected in EDTA tubes that were placed on ice. The rats were sacrificed by severing their hearts. Their livers were immediately excised and weighed. Smaller sections were cut from the liver samples to be used for biopsy. As for the larger sections, they were kept for fat extraction. Rat weights were recorded once after anesthetizing and another time after blood draw and liver excision.

The liver samples were frozen in liquid nitrogen, then stored at -80°C pending analysis. Blood samples were then centrifuged at 3500 (g) for 15minutes at 3°C and plasma aliquots were collected and stored at -80°C pending analysis. The rat carcasses were stored at -20°C pending drying, homogenization, and fat extraction.

B. Body Composition Analysis

The rat carcasses were dried in a convection oven for 48 hours at 105^0C. Weighing was done before and after drying. The rats were then homogenized and reflux fat extraction was done for 5 hours on the carcasses using petroleum ether solvent (BP 40-60^0 C). The carcasses were weighed before and after extraction. The water weight and body fat weight was estimated by calculating the weight differences.

C. Blood Analysis

1. Plasma Glucose, lipid profile, and phosphorous analysis

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).

2. Plasma Insulin

Plasma insulin concentration was determined by enzyme immunoassay using an insulin ELISA kit (EZRMI-13K) provided my EMD Millipore Corporation, USA.

D. Liver Analysis

1. Hepatic Fat Extraction

The larger liver sections were weighed before and after freeze-drying (2.5 Liter Bench top Freeze-Dry System, LABCONCO). After freeze-drying for 48 hours, the liver sections were crushed. Fat extraction was then performed on liver samples, where 1 gram of each sample was placed in moisture free sealable filter bags and weighed before and after extraction. Extraction was done for 40 minutes per run using petroleum

ether solvent (BP 40^0-60^0C) in Ankom XT10 (USA). Fat weight was calculated by subtracting the weight difference in the samples before and after fat extraction.

2. Liver Biopsy

The smaller liver sections were stained with hematoxylin and eosin (H&E) and oil red (O) for the evaluation of necro-inflammatory grading and fatty droplets. Histological changes were assessed by a modification of the scoring system for grading and staging for NAFLD described by (Kleiner et al., 2005).

3. Histopathology Examination

Rat liver tissue was processed into 3-4 um 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). Portal inflammation, fibrosis, liver cell ballooning; Mallory hyaline bodies and glycogenated nuclei were all absent.

4. Oil Red O (ORO) Examination

ORO was performed according to the described protocol (Mehlem et al., 2013). Briefly fresh frozen rat liver tissue was embedded into cryomolds and sectioned into 5 um 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.

E. Statistical Analysis

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

CHAPTER III

RESULTS

A. Body Weight and Food intake

1. Final weight and weight gain

The results for each group were expressed as Mean ± SD. The mean final body weight of each group was recorded on the 9th week before sacrifice. The average mean weight of the control group was significantly higher than the mean final weight of the 0.015% P group. Furthermore the mean final body weight of the control group was the highest among all the rat groups. However, amongst the low protein groups, as the P level in the rat groups increased, there was a significant increase in the mean final weight. Where the 0.3% P group had the highest mean weight amongst the low protein groups, and it was not significantly different from that of the control group, whereas the 0.015% P group had the lowest mean weight. (Figure 5)

Similarly, the average weight gain in grams per day over the 9-week period of the control group was almost twice greater than the low protein 0.015% group. (Figure 6) Thus the mean weight gain per day of the control group was the highest amongst all the groups. However, amongst the low protein groups, the addition of P showed a statistically significant improvement in the mean weight gain per day over the 9-week period. In which the 0.3% P group was not significantly different from the control group (Figure 6). A similar distinction was seen between the rat groups when the mean weight gain per day was assessed on a weekly basis. (Table 6)

Values with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).

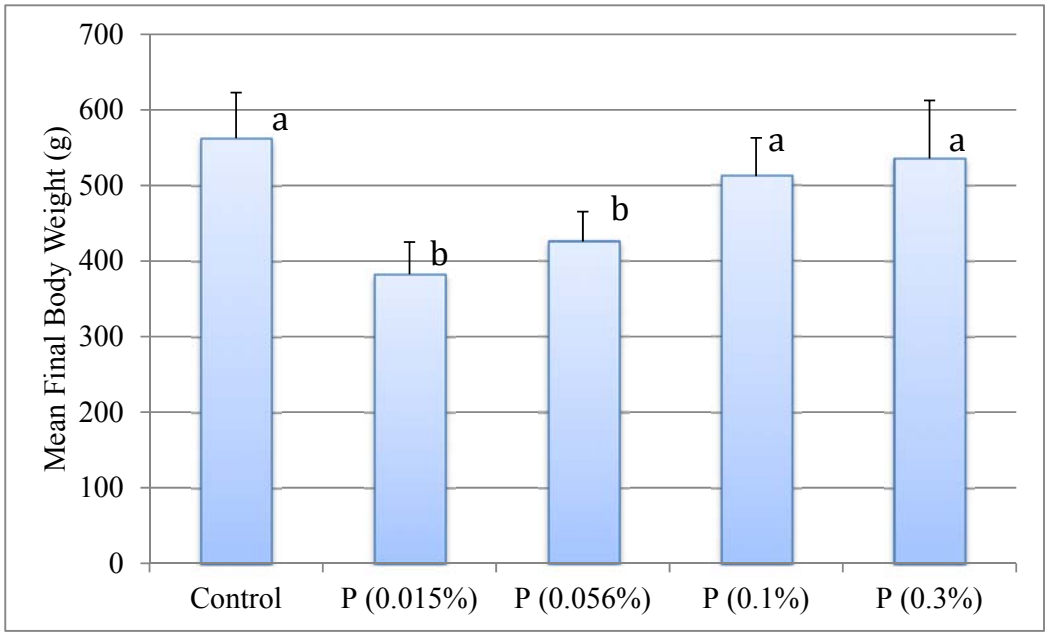

Figure 5: Mean Final Body Weight (g) of rat groups over 9-week period

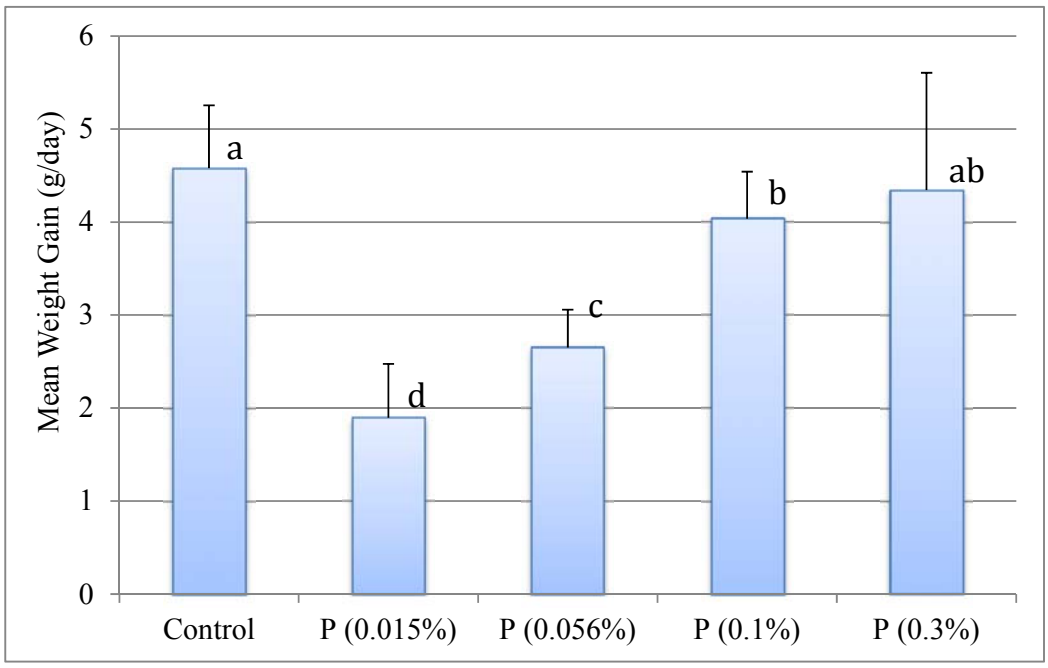

Figure 6: Mean Weight Gain (g/day) of rat groups over 9-week period

Table 6: Average Weight gain in grams per day of rat groups presented weekly.

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Anova P-value
Week 1 (g/day)	12.81 ± 1.91 [a]	5.01 ± 0.88 [d]	5.70 ± 1.12 [d]	8.24 ± 1.74 [c]	10.80 ± 2.97 [b]	0.000
Week 2 (g/day)	9.40 ± 1.49 [a]	3.28 ± 1.17 [d]	3.64 ± 1.05 [cd]	4.73 ± 0.72 [c]	7.00 ± 3.04 [b]	0.000
Week 3 (g/day)	8.1 ±1.28 [a]	5.19 ± 0.65 [b]	5.02 ±1.23 [b]	7.02 ± 1.66 [a]	4.98 ± 1.29 [b]	0.000
Week 5 (g/day)	5.49 ±1.45 [a]	2.99 ±1.56 [b]	4.51 ±1.46 [a]	4.72 ±1.21 [a]	5.72 ± 2.63 [a]	0.001
Week 6 (g/day)	0.69 ±1.27	0.31 ±1.39	0.99 ±1.28	1.93 ± 0.91	1.27 ± 1.73	0.160
Week 8 (g/day)	2.96 ±1.83 [a]	0.61 ±0.93 [b]	1.79 ±1.06 [ab]	2.79 ±1.119 [a]	2.11± 2.05 [a]	0.008
Week 9 (g/day)	1.68 ± 1.02 [bc]	0.88 ± 0.45 [c]	1.73± 0.77 [b]	2.77 ± 1.22 [a]	1.94 ± 0.83 [b]	0.001

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).
Data from weeks 4 and week 7 was not shown due to an error in the balance.

2. Food Intakes and Energy Efficiency

Average food intake in grams per day of each group was expressed as Mean ± SD. The mean food intake per day of the control group was significantly higher than the 0.015% P group. Moreover, among the low protein groups the addition of P showed a statistically significant improvement in the mean food intake per day over the 9-week period. In which the 0.3% P group was not significantly different from the control group. (Figure 7) A similar distinction in food intake was seen between the rat groups when the mean intake per day was assessed on a weekly basis. (Table 7)

The mean energy efficiency EE of each group was calculated in weight gained in grams per 100 kcal of energy consumed from the diet. The results showed the average energy efficiency of the control group was almost 2-folds greater than the 0.015%P group. Nevertheless, when comparing the low protein groups, the mean EE was directly related to P level (Figure 8). In which the increase in P level was associated with an increase in the energy efficiency. The 0.3% P group had the highest energy efficiency amongst the low protein groups, thus resembling that of the control group which had the highest energy efficiency amongst all groups. However, the 0.015% P group had the lowest EE. A similar distinction in EE was seen between the rat groups when it was assessed on a weekly basis. (Table 8)

Values with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).

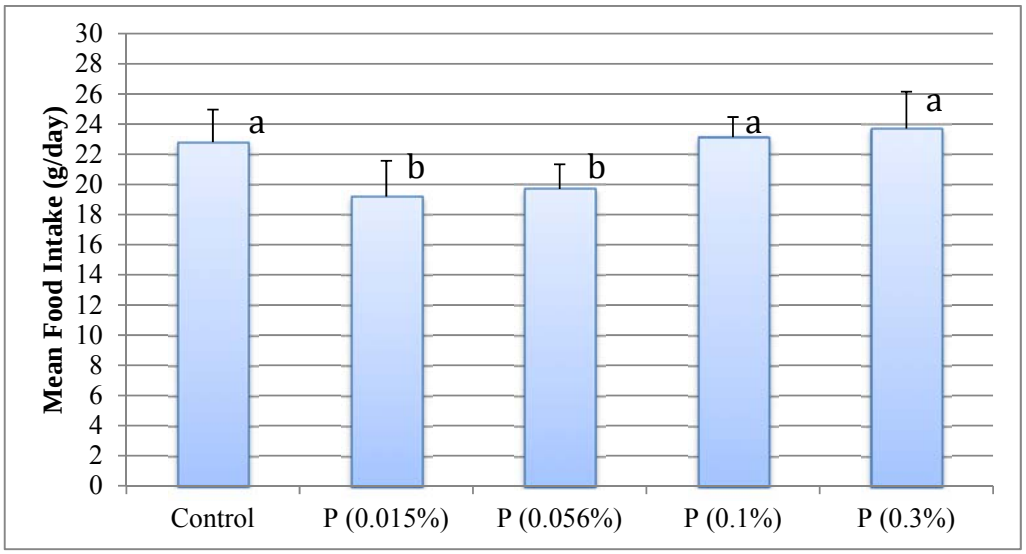

Figure 7: Mean food intake in grams per day of rat groups over the 9-week period

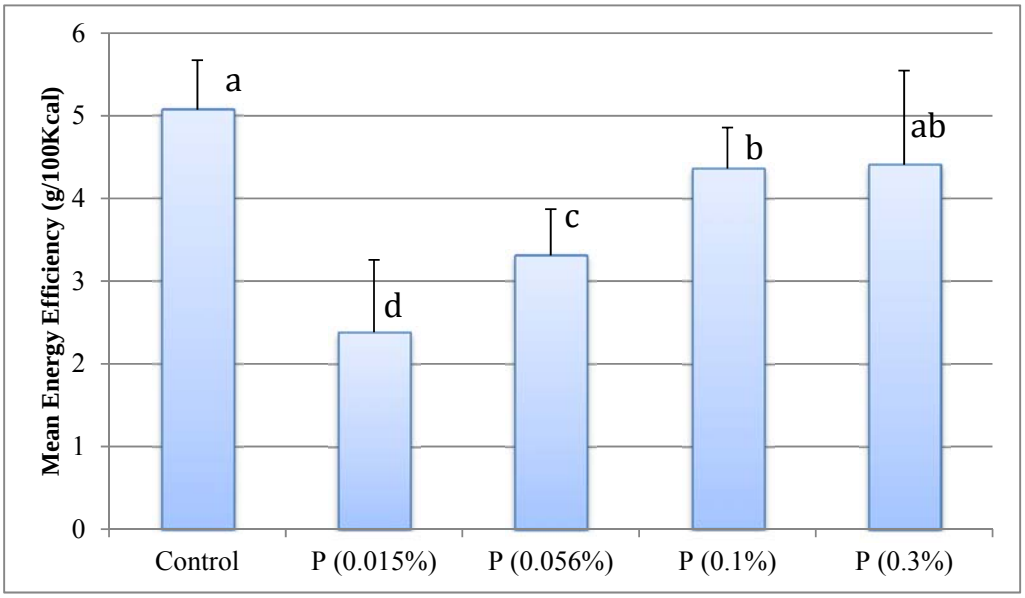

Figure 8: Mean energy efficiency in grams per day of rat groups over the 9-week period

Table 7: Average food intakes in grams per day of rat groups presented weekly.

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Anova P-value
Week 1 (g/day)	21.96 ± 3.39	22.13 ± 8.81	18.49 ± 2.83	21.01 ± 4.23	23.51 ± 3.44	0.239
Week 2 (g/day)	22.58 ± 2.32 [a]	17.62 ± 2.63 [c]	18.61 ± 2.31 [c]	19.83 ± 2.59 [bc]	22.03 ± 3.4 [ab]	0.001
Week 3 (g/day)	22.78 ± 2.49 [a]	18.35 ± 3.86 [b]	18.95 ± 3.63 [b]	23.80 ± 2.847 [a]	24.19 ± 2.967 [a]	0.000
Week 5 (g/day)	25.91 ± 2.81 [a]	21.89 ± 2.86 [b]	21.70 ± 2.97 [b]	26.11 ± 2.30 [a]	28.00 ± 4.12 [a]	0.000
Week 6 (g/day)	25.60 ± 4.00 [a]	20.86 ± 3.51 [b]	20.79 ± 2.92 [b]	25.16 ± 2.96 [a]	25.84 ± 3.24 [a]	0.001
Week 8 (g/day)	23.84 ± 3.24 [a]	18.30 ± 3.07 [b]	19.27 ± 3.119 [b]	24.07 ± 5.36 [a]	23.57 ± 3.71 [a]	0.002
Week 9 (g/day)	22.64 ± 2.805 [a]	18.14 ± 2.64 [b]	19.63 ± 1.46 [b]	24.10 ± 2.22 [a]	22.87 ± 3.25 [a]	0.000

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).
Data from week 4 and week 7 was not shown due to an error in the balance.

Table 8: Average EE in grams per 100 Kcal of rat groups presented weekly.

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Week 1 (g/100Kcal)	11.45 ± 1.98 ^{a}	4.10 ± 1.83 ^{c}	4.36 ± 0.84 ^{c}	6.97 ± 1.83 ^{b}	10.56 ± 1.86 ^{a}	0.000
Week 2 (g/100Kcal)	8.68 ± 1.59 ^{a}	2.38 ± 1.41 ^{c}	2.82 ± 1.16 ^{c}	3.83 ± 0.79 ^{c}	6.52 ± 2.21 ^{b}	0.000
Week 3 (g/100Kcal)	7.57 ± 0.71 ^{a}	3.84 ± 0.85 ^{b}	3.81 ± 1.80 ^{b}	6.92 ± 0.95 ^{a}	4.90 ± 1.41 ^{b}	0.000
Week 5 (g/100Kcal)	5.86 ± 1.06 ^{ab}	2.73 ± 1.64 ^{d}	4.09 ± 1.16 ^{cd}	5.10 ± 0.85 ^{bc}	6.69 ± 1.84 ^{a}	0.000
Week 6 (g/100Kcal)	0.80 ± 1.33	0.41 ± 1.81	0.89 ± 1.43	1.93 ± 0.96	1.34 ± 1.61	0.145
Week 8 (g/100Kcal)	3.01 ± 1.50 ^{a}	0.56 ± 1.46 ^{c}	1.50 ± 1.37 ^{bc}	2.79 ± 0.87 ^{ab}	2.20 ± 1.83 ^{ab}	0.005
Week 9 (g/100Kcal)	1.63 ± 0.86 ^{b}	0.68 ± 0.55 ^{c}	1.37 ± 1.01 ^{bc}	2.77 ± 1.14 ^{a}	1.83 ± 0.79 ^{b}	0.000

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).
Data from weeks 4 and week 7 was not shown due to an error in the balance

B. Body Composition

There was a statistically significant difference ($p<0.05$) in the mean wet and the mean dry weight between the rat groups in which the control group had the highest mean wet weight (507.4g) and dry weight (230.4g). (Table 9) When comparing between the low protein groups, the 0.015%P group had the lowest mean weight. The mean weight increased as the P level of the group was higher, making the 0.3%P group the highest amongst the low protein groups. (Table 9)

Similarly, there was a significant difference ($p<0.05$) in the mean water weight of the rat groups. The control group had the highest weight between all the groups whereas the 0.1% P group had the highest mean water weight (261.1g) between the low protein groups. (Table 9) A similar significant pattern was observed amongst the groups when comparing the fat weight, and the defatted weight calculated from both the dry and wet weights of the rats.

However, there was no significant difference observed in the mean percentage of fat between the rat groups. Furthermore, there was no significant difference in the mean percentage defatted weight calculated from both dry and wet weight. (Table 10)

Conversely, there was a significant difference in the mean percentage of water between the groups. The 0.015% group had the highest percentage (65%) ($P<0.05$) among all the rat groups. As the P level amongst the low protein groups was higher the percentage water decreased in which the 0.3%P group had the lowest mean percentage (53.8%) and it was not significantly different from the control group. (Table 10)

Table 9: Mean water, fat, and defatted weight (g) per rat groups

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Wet Weight (g)	507.4 ± 56.0 [a]	343.8 ± 42.5 [b]	381.0 ± 38.5 [b]	460.3 ± 50.0 [a]	481.6 ± 73.6 [a]	0.000
Dry Weight (g)	230.4 ± 50.0 [a]	119.4 ± 18.8 [b]	143.9 ± 26.1 [b]	199.2 ± 48.6 [a]	228.1 ± 80.9 [a]	0.000
Water Weight (g)	277.1 ± 19.5 [a]	224.4 ± 25.6 [d]	237.1 ± 21.4 [cd]	261.1 ± 22.4 [ab]	253.5 ± 17.8 [bc]	0.000
Fat Weight (g)	56.4 ± 21.2 [ab]	24.9 ± 9.4 [c]	33.8 ± 15.8 [bc]	56.6 ± 32.1 [ab]	64.9 ± 37.7 [a]	0.006
Defatted from dry (g)	174.0 ± 33.7 [a]	94.5 ± 9.9 [c]	110.1 ± 14.9 [c]	142.7 ± 22.5 [b]	163.2 ± 46.7 [ab]	0.000
Defatted from wet (g)	451.1 ± 41.4 [a]	318.8 ± 34.5 [c]	347.2 ± 32.9 [c]	403.7 ± 32.0 [b]	416.7 ± 41.3 [b]	0.000

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).

Table 10: Mean percentage of water, fat, and defatted per rat groups

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Water (%)	55.05 ± 5.56 ^{c}	65.358 ± 2.048 ^{a}	62.40 ± 3.74 ^{ab}	57.17 ± 6.30 ^{bc}	53.88 ± 9.70 ^{c}	0.000
Fat (%)	24.20 ± 5.34	20.61 ± 4.99	23.13 ± 6.11	27.31 ± 8.64	26.66 ± 8.52	0.230
Defatted from dry (%)	76.07 ± 5.26	79.74 ± 4.89	77.11 ± 5.97	73.05 ± 8.49	73.66 ± 8.43	0.220
Defatted from wet (%)	89.11± 3.30	92.912 ± 2.02	91.23 ± 3.30	88.09 ± 5.52	87.24 ± 6.09	0.042

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).

C. Plasma Results

1. Lipid Profile

There was no significant difference in the lipid profile parameters between the rat groups. However the mean cholesterol and mean HDL levels of the control group seemed to be higher than the low protein groups. However P manipulation in the diet did not show statistically significant discrepancies in plasma cholesterol, triglyceride, HDL and LDL levels between the rat groups. (Table 11)

Table 11: Mean plasma cholesterol, triglyceride, LDL, and HDL per rat group

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean± SD	
Cholesterol (mg/dl)	105.40 ±25.26	87.00 ± 22.09	85.30 ±13.31	84.80 ± 14.71	85.5 ±15.9	0.081
TG (mg/dl)	41.20 ± 13.07	35.67 ± 6.69	41.20 ± 17.24	45.00 ± 22.68	40.60 ± 15.77	0.807
LDL (mg/dl)	26.20 ± 8.02	23.22 ± 6.28	21.60 ± 3.78	19.80 ± 5.09	20.22 ± 4.02	0.103
HDL (mg/dl)	71.20 ± 16.35[a]	56.6 ± 15.9 [ab]	55.4 ± 10.48 [ab]	55.9 ± 12.2 [ab]	54.70 ± 8.10 [b]	0.031

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).

2. Glucose, Phosphorus, and Insulin

Mean plasma glucose concentration was the highest in the control group and it was approximately two-folds greater than the 0.015%P group. However, amongst the low protein groups, the mean plasma glucose concentration increased with the increase in P level of the rat groups. The 0.3%P group had the highest glucose concentration among the low protein groups ($P<0.05$), thus resembling the control group. (Table 12)

Mean plasma phosphorus concentration was the lowest in the 0.015%P group, however no significant difference in the mean plasma phosphorus was observed between the other groups.

Additionally, mean insulin concentration was not significantly different between the rat groups. However, a trend was observed between the low P groups where there was a slight increase in the mean insulin concentration as the P level increased. Also a bigger difference in the mean insulin concentration can be seen between the control group and the 0.015%P group. (Table 12)

3. Albumin and CRP

There is no statistically significant difference observed in plasma albumin and CRP. (Table 13)

Table 12: Mean plasma glucose, phosphorus, and insulin per rat group

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Glucose (mg/dl)	277 ± 76.5 [a]	148.3 ± 91.7 [b]	205 ± 57.5 [ab]	245.4 ± 83.3 [ab]	263.9 ± 68.6 [a]	0.004
Phosphorus (mg/dl)	6.36 ± 0.86 [ab]	5.41 ± 0.91 [b]	6.45 ± 1.23 [ab]	7.37 ± 0.86 [a]	6.37 ± 0.74 [ab]	0.002
Insulin (ng/ml)	0.74 ± 0.94	0.38 ± 0.18	0.47 ± 0.39	0.84 ± 0.76	0.82 ± 0.89	0.487

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05)

Table 13: Mean plasma Albumin and CRP per rat group

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Albumin (g/dl)	3.15 ± 0.25	3.16±0.22	3.15±0.26	3.11±0.23	3.07±0.24	0.921
CRP	3.1 ± 0.32	3.22 ± 0.44	2.8 ± 0.42	2.7 ± 0.68	2.7 ± 0.82	0.162

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05)

D. Liver Results

1. Liver Weight and Fat Content

Mean liver weight and mean liver dry weight were significantly higher in the control groups when compared with the 0.015% P group. However, the weights increased in the low protein groups with the increase in dietary P. (Table 14)

Nevertheless there was no significant difference in the mean liver weight per 100 gram of rat body weight between the groups. This shows that the changes in liver weight are proportional to the changes in rat body weight. Likewise, there was no significant difference in the mean liver fat weight and the mean percentage of fat in the liver between the rat groups. (Table 14)

2. Histology

No significant difference was noted across groups regarding steatosis grade, location, portal and lobular inflammation and micro vesicular steatosis. (Table 15)

All the groups had approximately a similar number of cases of microvesicular steatosis. Around 5 to 6 rats from each group presented with Grade 0 and Grade 1 steatosis, and only 1 rat from each group developed Grade 3 steatosis. There were no signs of mallory hyaline, fibrosis, and glycogenated nuclei. Therefore, the overall distinctive features of NAFLD were not present. So the effect of P manipulation on the development of NAFLD could not be explored.

Table 14: Mean liver weight and fat content per rat group

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Liver wt (g)	16.31 ± 2.25 [a]	11.99 ± 2.11[b]	12.48 ± 1.89 [b]	15.31 ± 2.42 [a]	16.36 ± 3.32 [a]	0.000
Liver Dry wt (g)	4.77 ± 0.64 [a]	3.32 ± 0.48 [b]	3.59 ± 0.53 [b]	4.51 ± 0.73 [a]	4.80 ± 1.09 [a]	0.000
Liver wt (g/100g)	3.09 ± 0.24	3.29 ± 0.36	3.11 ± 0.31	3.19 ± 0.40	3.24 ± 0.24	0.545
Liver Fat wt (g)	0.53 ± 0.17	0.27 ± 0.08	0.47 ± 0.24	0.50 ± 0.21	0.62 ± 0.49	0.092
Liver Dry wt %	70.74 ±1.04	72.15 ±1.50	71.13 ± 2.21	70.52 ± 1.55	70.74 ± 1.26	0.187
Liver Fat %	11.03 ± 2.70	7.99 ± 1.96	13.06 ± 5.88	11.03 ± 3.56	11.93 ± 6.46	0.186

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).

Table 15: Liver Histology

	(Control) N=10	0.015% P N=9	0.056% P N=10	0.1% P N=10	0.3% P N=10	Anova P-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Oil-R-O Image Analysis	664 ±1457	280 ± 727	1510 ± 2837	1234 ± 1636	2075 ± 3088	0.407
Steatosis Grade	0.3 ± 0.68	0.4 ± 1.01	0.5 ± 0.97	0.9 ± 1.1	0.6 ± 0.97	0.701
Location	0.3 ± 0.94	0.68 ± 1.0	0.7 ± 1.25	1.8 ± 1.55	0.9 ± 1.45	0.118
Micro vesicular steatosis	0.5 ± 0.53	0.56 ± 0.53	0.6 ± 0.52	0.9 ± 0.32	0.7 ± 0.48	0.385
Lobular inflammation	0.1 ± 0.32^{b}	0.67 ± 0.87^{ab}	0.7 ± 0.95^{ab}	1.4 ± 1.08^{a}	0.5 ± 0.71^{b}	0.020
Portal inflammation	0.1 ± 0.32	0.22 ± 0.44	0.20 ± 0.42	0.10 ± 0.32	0.10 ± 0.32	0.895
Mallory hyaline	0	0	0	0	0	-
Fibrosis	0	0	0	0	0	-
Glycogenated nuclei	0	0	0	0	0	-

Values in the same row with different superscripts are significantly different based on Fisher's pair wise comparison (P<0.05).

CHAPTER IV

DISCUSSION

The distinction in the mean final body weight between the rat groups as well as the mean weight gain per day observed in Figures 5 and 6 respectively is attributed to the changes in food intake and energy efficiency. This relation can be seen when the mean energy efficiency was calculated, which represents the grams of weight gained in every 100 Kcal consumed. The rat groups showed a similar pattern in their weight changes, food intakes, and energy efficiency. The food intake was the highest in the control group receiving a normal protein (20%) and normal P (0.3%) concentration in the diet; hence the mean weight gain and mean final body weight were also the highest. On the other hand, the lowest food intake and as a result the lowest weight gain was observed in the low protein (10%) low P (0.015%) group. When comparing the control group with the low protein groups, looking at protein alone as a variable, it can be observed that the rats in the low protein groups had adapted to the diet by down regulating their food intake. This adaptation as a response to a low protein diet has been detected in previous literature (Kabadi et al., 1976; Peters & Harper, 1985; Heard et al., 1977).

However, the objective of this experiment was to dissect the effect of dietary phosphorus from that of protein. Therefore, it is important to note that when examining the low protein groups alone, the diets given to the 4 groups were similar in composition and were iso-caloric. Thus the only variable was the percentage of phosphorus. There was a clear and significant difference in food intake between the low

protein groups in which the increase of P level in the diet was directly related to the increase in food intake. According to Radcliffe & Webster (1976), an animal is capable of regulating its food intake in order to serve two goals. The first goal is to satisfy its nutritional needs based on its stage of growth and maturity, and this requires long-term regulation of intake. The second goal is to maintain homeostasis, and this requires short-term regulation of food intake (Radcliffe & Webster, 1976). In the case of our experiment it is postulated that the rats were regulating their food intake in order to maintain homeostasis.

It is well known that the uneven supply of essential amino acids in the diet leads to inadequate growth. Consequently, several studies examined the effect of varying dietary protein concentrations on amino acid homeostasis. Focusing further on the rapid adaptation of the rat to avoid consuming an amino acid imbalanced diet (Leung et al., 1968; Sanahuja et al. 1963) The rats in these cases were hypothesized to be regulating their food intake in order to avoid toxicity from a specific amino acid (Harper et al., 1970; Morrison et al., 2012). The results of our experiment could not be attributed to this hypothesis since dried egg white was used as the exclusive source of protein in the diet, which is known to be a complete protein providing all the essential amino acids; thus making phosphorus the only variable influencing food intake in this experiment.

Nevertheless, there is no disregard of the evidence that protein and amino acid homeostasis is the main mechanism affecting food intake in rats. This suggests that dietary P has a strong influence on the metabolic homeostasis of protein. Regulation of free amino acids occurs on multiple levels. Proteolysis, and the breakdown of dietary protein increase the amino acid pool. On the other hand, amino acids are utilized for

protein synthesis, synthesis of glucose, from gluconeogenesis, and the biosynthesis of nitrogen compounds. Moreover, excess amino acids are catabolized and directed into urea synthesis (Harper et al. 1970). Protein and urea synthesis are important metabolic pathways required for utilizing and excreting excess amino acids respectively. However, these processes are ATP dependent. Furthermore, it is known that amino acids can be oxidatively converted to glucose via gluconeogenesis. This process leads to ATP production that is very nearly equal to the ATP required to convert the amino acid carbon skeleton to glucose (Jungas et al., 1992).

The rate of protein turnover in humans is equivalent to 300 to 400g/day. On the other hand, the average daily protein intake in regular healthy adults ranges between 50 to 80g/day (Schutz, 2011). Protein synthesis is known to be energy expensive, in which 4 ATP equivalents are required for the formation of 1 peptide bond, or the equivalent of 0.67 Kcal per one gram of protein synthesized (Bender, 2012).

ATP production in the body is directly related to the availability of phosphorus in the circulation. Therefore, an adequate dietary supply of phosphorus is of paramount importance. A study was done on mice to test the effect of dietary phosphorus deficiency on ATP synthesis in the muscle, the results showed that ATP production was slower in the phosphate deficient group of mice during muscle contraction. Furthermore, the rate of ATP restoration during the recovery phase was also slower in the phosphate-deficient group (0.09%P) when compared with the control (0.6% P) (Hettleman et al., 1983).

Dietary P depletion may lead to ATP depletion, making it unavailable for protein and urea synthesis. Furthermore, studies done on both rat and human subjects

have reported an adaptive reduction in amino acid oxidation as a response to inadequate protein intake (Young & Marchini, 1990). This in turn may lead to the accumulation of free amino acids in the circulation. The accumulation of amino acids will signal a homeostatic response causing it to decrease its food intake. This hypothesis can explain the improvement in food intake and weight gain as the P level in the diet increased. The 0.3% P group had approximately similar intake and weight gain as the control group, whereas the 0.015%P group had the lowest food intake and weight gain. This could mean that adequate dietary P may play a role in facilitating amino acid metabolism making the diet for the rat more balanced and consequently more desirable.

The rat groups did not show significant differences in the mean percentage of fat and mean percentage defatted weight in their composition. These results imply that the rat groups had similar composition of fat and lean mass regardless of the dietary changes in protein and/or phosphorus. However, there is a significant difference in the percentage of water between the rat groups. As the P level amongst the low protein groups was higher the percentage water decreased in which the 0.3% P group had the lowest mean percentage resembling the control group. One way to explain this observation is to refer to the etiology of edematous malnutrition or Kwashiorkor. There is controversy pertaining to the etiology of edema in Kwashiorkor. Evidence has shown that dietary protein is not the only factor contributing to the development of water retention and edema. A study of six different diets given to 103 children with edematous malnutrition showed that the rate of loss of edema was strongly correlated with the dietary energy intake but not with the protein intake. (Golden, 1982)

It is important to note that Kwashiorkor is most prevalent in populations whose

diets are based on maize, cassava, or rice (Manary et al., 2009). These dietary components are known to be poor dietary sources of phosphorus due to its low bioavailability for absorption. For instance, around 70% of total phosphorus in maize is bound in the form of phytate phosphorus (Sands et al., 2001). Phytate-phosphorus cannot be hydrolyzed and absorbed by humans, and monogastric animals, since they lack the enzyme phytase. A study done on pigs aimed to compare the bioavailability of phosphorus in normal corn versus high available phosphorus corn (HAP), they also aimed to test the efficacy of phytase for improving growth performance and nutrient balance. The results showed that pigs maintained on normal corn had the slowest rate of weight gain and the lowest body weight ($p<0.05$). Furthermore, the feed efficiency measured was lower in the normal corn diet when compared to the HAP corn diet. (Sands et al. 2001) Therefore, we can hypothesize that the increased food intake in the rat group receiving the highest P level (0.3%) in its diet is correlated with the decrease in the mean percentage of water in their body composition. This could be an indicator of an improvement in body composition, as well as a decrease in the risk of developing symptoms of Kwashiorkor while maintained on a low protein diet.

This study aimed to examine the effect of phosphorus manipulation on the development of non-alcoholic fatty liver disease. Six-week old male Sprague-Dawley rats were maintained on a low protein diet for 9 weeks in an attempt to induce NAFLD. However, this study showed that maintaining Sprague-Dawley strains on a low protein diet for 9 weeks did not induce NAFLD, so the effect of phosphorus on the progression of the disease could not be assessed.

The most commonly used animal models to study NAFLD include rats and

mice. More specifically, genetic models, high-fat diets, or choline-methionine deficient diets were most frequently observed in the literature. In some of these models, the use of Sprague-Dawley rat strain was common. Hepatic steatosis was observed in these models along with some histologic features of steatohepatitis, though fibrosis was rarely present. (Kucera & Cervinkova 2014) However, to our knowledge, very few animal models were developed to assess NAFLD in the specific case of dietary protein depletion. Also no previous study examined the exclusive role of dietary phosphorus level on ATP depletion, which consequently might lead to the development of fatty liver, as it is observed in many cases of Kwashiorkor. According to (Kucera & Cervinkova 2014) the ideal animal model for NAFLD should reflect all the complex features of the disease.

Furthermore, in this study, protein was only restricted to 10% of total caloric intake, although it is considered to be a low-protein diet, it is possible that this restriction was not severe enough to induce the symptoms of Kwashiorkor. We can postulate that a further restriction of protein to 8% or 5% of total caloric intake may lead to the development of fatty liver. Also, it is possible that the duration of the experiment may play a role in the induction of the disease, where maintaining the rats on a protein-restricted diet for more than 9 weeks may induce fatty liver. Kwashiorkor is known to be a very severe form of protein malnutrition leading to mortality in children, so it is sensible to assume that a more severe dietary restriction may lead to the development of more adverse symptoms in the rats. Additionally, a common rat strain used to examine NAFLD is the Wistar rat strain, which was proven to be successful in inducing fatty liver. Therefore, taking into consideration the Wistar rat strain in future research may enable a more successful representation of the features of NAFLD.

Therefore, further research should be done and more experiments should be made in order to develop a more successful model to assess the progression of NAFLD in the case of a low protein diet. Thus, choosing the most appropriate model while taking into consideration its limitations, remains to be the best way to further explore this disease.

The limitation of this study was an error that occurred in the weighing of the rats and their food intakes during weeks 4 and 7. In these two weeks, weighing was only performed once instead of twice per week due to an error that had occurred in the balance. This lead to insignificant differences in the values between the rat groups during these 2 weeks. However, the values in these 2 weeks were included in the calculation of the mean weight gain, food intake, and energy efficiency measured over the 9-week period, and they had no negative influence on the statistical significance of the results.

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

The purpose of this study was to use 49 male Sprague-Dawley rats in order to dissect the exclusive role of varying concentrations of dietary phosphorus on weight gain, food intake, body composition, serum lipid profile, serum glucose, serum insulin levels, and liver fat content in rats maintained on a low protein diet for 9 weeks.

The results of the experiment showed that the different rat groups had a similar pattern in the difference in their weight changes, food intakes, and energy efficiency. It can be observed that the rats in the low protein low P groups had adapted to the diet by down regulating their food intake. This adaptation in food intake was hypothesized to be due to the depletion of dietary P, which in turn may lead to ATP depletion, making it unavailable for protein and urea synthesis, which are energy expensive metabolic processes. Thus the accumulation of amino acids will signal a homeostatic response in the rat causing it to decrease its food intake.

There was a significant difference in the percentage of water between the rat groups. As the P level amongst the low protein groups was higher the percentage water decreased. This result could be an indicator of an improvement in body composition in the higher P group due to the improvement in food intake. Hence, adequate dietary P may be decreasing the risk of developing symptoms of Kwashiorkor while maintained on a low protein diet.

Maintaining Sprague-Dawley strains on a low protein diet for 9 weeks did not induce NAFLD, so the effect of phosphorus on the progression of the disease could not

be assessed. Therefore, further research should be done and more experiments should be made in order to develop a more successful model to assess the progression of NAFLD in the case of a low protein diet.

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