Phosphorus supplementation mitigated food intake and growth of rats fed a low protein diet

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Abbreviations include:
ATP: Adenosine Triphosphate;
EDTA: Ethylenediaminetetraacetic acid;
HDL: High density lipoprotein;
LDL: Low density lipoprotein.
NAFLD: Nonalcoholic fatty liver disease;
P: Phosphorus;
The authors declare no conflict of interest.
Abstract

**Background:** Low protein intake is associated with various negative health outcomes at any stage of life. When diets do not contain sufficient protein, phosphorus (P) availability is compromised since proteins are the major sources of P. However, whether mineral phosphorus supplementation mitigates the problem is unknown.

**Objective:** Our goal was to determine the impact of dietary P supplementation on food intake, weight gain, energy efficiency, body composition, blood metabolites and liver histology of rats fed a low protein diet for 9 weeks.

**Methods:** Six-week old male Sprague-Dawley rats (n=49) were randomly allocated to 5 groups and fed ad-libitum 5 iso-caloric diets that only varied in protein (egg white) and P concentrations for 9 weeks. The control group received 20% protein diet (normal P, 0.3%), whereas the 4 other groups were fed low protein (10%) diets with various P concentrations: 0.015%, 0.056%, 0.1% and 0.3%. The rats’ weights, body and liver composition, and plasma biomarkers were then assessed.

**Results:** Food intake, weight gain and energy efficiency increased significantly with the addition of P to the low protein diet and were similar among the 0.3% P groups (LP-0.3P and NP-0.3P) regardless of their dietary protein content. Additionally, P supplementation of the low protein diets was observed to reduce plasma urea nitrogen and to increase total body protein content (defatted). Changes in food intake and efficiency, body weight and composition and plasma urea content were highly pronounced at a dietary P level below 0.1%, which may represent a critical threshold.

**Conclusion:** In rats, the addition of P to low protein diets improved growth measures mainly due to an enhancement in energy efficiency. Dietary P level of 0.3% mitigated detrimental effects of low protein diets on growth parameters.
Keywords: low protein diet, phosphorus, weight gain, food intake, energy efficiency,

NAFLD, Sprague-Dawley rats
Introduction

Protein deficiency can lead to various adverse health effects at all stages of life. Protein restriction compromises growth and muscle maintenance of adolescents and leads to weight loss, reduced subcutaneous fat, increased susceptibility to infection, general lethargy and delayed wound healing in adults [1]. Additionally, inadequate protein content of maternal diets during pregnancy, was reported to result in negative health outcomes in offspring [2]. Animal experiments on rodents have shown that maternal low protein diet during pregnancy is associated with disproportionate patterns of fetal growth [3], increased adult onset of metabolic disorders, and increased adiposity and glucose intolerance [4]. Furthermore, low dietary protein intake in rodents has been consistently found to be associated with increased body fat content [5-8].

The metabolic response of animals to protein restriction is believed to be mediated via alterations in food intake [9, 10], organ size and enzymatic function [11, 12]. Though changes in food intake were reported to be inconsistent, where some studies showed an increase [7, 8, 13], while others reported a decrease that was dependent on the extent of protein restriction [10, 14]. This inconsistency may imply that other factors are involved in the regulation of food intake. Although proteins vary in P content and bioavailability [15], the synergistic relationship between dietary protein and P intake, suggest that dietary P modulates the effects of protein restriction. P is an essential mineral and thus plays an important role in cellular metabolism. It is also integrated within adenosine triphosphate (ATP), which acts as a phosphate donor for many metabolic reactions [16]. Such reactions are strongly dependent on body P availability for its production [17, 18] and that is obviously related to dietary P availability.

Populations most at risk of developing diseases associated with protein malnutrition, such as Marasmus and Kwashiorkor, are those whose staple foods are low in protein quantity and
quality (e.g. cassava, maize, rice) [19]. A consequence of low protein diets is low P diets which tends to compound nutritional problems. These populations also consume minimal amounts of animal and dairy products, which are known to be the most important dietary sources of both high quality proteins and P. In contrast, most stable plant-based foods, such as whole wheat or brown rice; that are also commonly consumed by at-risk populations, they are of low protein quantity and quality which contain low amounts of bioavailable P, as P is mainly bound in the form of phytate that makes it unavailable for absorption [20].

Fermentation is known to breakdown phytate and thus improves the bioavailability of P [21]; yeast fermentation for about 60 min was reported to reduce phytate content of bread by about 10% [22]. Under conditions of protein restriction, the intake of P is compromised and therefore it can be assumed that inadequate dietary P intake compounds the negative effects of protein restriction. Accordingly, the present study was performed to assess the impact of P on food intake, weight gain, food efficiency and body composition of rats maintained on a low protein diet with varied concentrations of dietary P.

Methods

Animal Housing

Forty-nine, six-week old male Sprague-Dawley rats (Animal care facility, American University of Beirut, Beirut, Lebanon) were housed individually in a temperature (22±1°C) and light (12:12 hours light/dark cycle, light on 0700 h) controlled room. The rats had free access to water and were fed ad libitum a semisynthetic powder control diet (Supplemental Table 1) for one week to familiarize them with the environment and the diet.

Experimental Diet
The semi-synthetic powder experimental diets (Supplemental Table 1) were all prepared using the same ingredients and were iso-caloric. Dried egg white was used as the main source of protein since it supplies all essential amino acids and contains negligible amounts of P (1.5±0.013 g/kg) [23]. Phosphorus free mineral mix (AIN-93G MIX without P) and potassium phosphate (KH₂PO₄) from Dyets Inc. (Bethlehem, Pennsylvania, USA) were used to modify P content of the diet. KH₂PO₄ was used because potassium addition does not affect growth [24, 25].

**Experimental Design**

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the American University of Beirut. Following the one-week acclimation period, each rat (190-315g) was randomly allocated to one of the five experimental groups. Under normal conditions, the recommended P content of the rats’ diet is 0.3% [26] and this was the dietary proportion used for the control group. Treatments were as follows (Supplemental Table 1):

- **Control Group**: 20% protein and 0.3% P
- **0.015% P Group**: 10% protein and 0.015% P
- **0.056% P Group**: 10% protein and 0.056% P
- **0.100% P Group**: 10% protein and 0.1% P
- **0.300% P Group**: 10% protein and 0.3% P

The rats were offered their corresponding diets ad libitum for 9 weeks. At termination, overnight fasted rats were anesthetized by isoflurane (Forane®, Abbott, Berks, UK) and blood was collected from the superior vena cava. The rats were then euthanized by removing the heart and their livers were extracted and weighed. Immediately afterword, two small liver sections were taken for histological analysis and the remaining section was frozen in liquid
nitrogen, and stored at -80°C. Blood samples were centrifuged at 2200 g for 15 minutes at 3°C and plasma aliquots were collected and stored at -80°C. The rat carcasses were stored at -20°C for body composition analysis.

Food Intake and Body Weight and Composition

The Food intake and body weight were measured twice per week and then averaged to calculate weekly changes. Carcasses were dried until constant weight (about 48 hours) at 105°C and homogenized, and the fat was extracted using petroleum Ether (BP 40-60°C).

Carcass moisture and fat contents were calculated from the differences in weight. Hepatic fat content was determined as follows: about 2g of liver were freeze-dried (2.5 Liter Bench top Freeze-Dry System, LABCONCO) for 48 hours and fat was extracted by petroleum ether solvent (BP 40-60°C) for 40 minutes using an ANKOMXT10 extractor (ANKOM Technology 2052 O'Neil Road, Macedon NY 14502).

Blood Analysis

Fasting plasma glucose, triglyceride, total cholesterol, and total P were determined using an enzymatic colorimetric method on the Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Johnson & Johnson, New York). Plasma insulin concentration was determined by enzyme immunoassay using insulin rat ELISA kit (EZRMI-13K) (EMD Millipore Corporation, Billerica, MA, USA).

Liver Biopsy

Small liver sections were stained with hematoxylin and eosin (H&E) and oil red (O) to be evaluated for Necro-Inflammatory grading and Fatty Droplets. Histological changes were
assessed by a modification of the scoring system for grading and staging for non-alcoholic fatty liver disease (NAFLD) as described by Kleiner et al. [27].

**Histopathology 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 semi-quantified 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).

**Oil Red O (ORO) Examination**

ORO was performed according to previously described protocol [28]. 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 semi-quantified using imagej 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, and image analysis for ORO surface area staining determined.

**Statistical Analysis**

The required number of rats (n=9) was based on previous weight gain data (6.0g/day SD 0.95) assuming a 25% difference in the mean with a statistical power of 90% and 5%
significance level. Data were expressed as means ± SD of all values. Data analysis was performed using the SPSS 23 (IBM SPSS) 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.

Results

Weight Gain, Food Intake and Energy Efficiency

Initial body weight was similar among the groups, while final body weight was significantly different. Final body weight of the control group (NP-0.3P) was significantly greater than those of the LP-0.015 P and LP-0.056 P groups. Among the low protein groups, body weight increased with increased P level in the diet, and the body weight of LP-0.1 P and LP-0.3 P groups were not significantly different from body weight of the NP-0.3P (Table 1).

Addition of P to low protein diets was found to increase weight gain (g/day), in which that of LP-0.1P and LP-0.3P was close to that of NP-0.3P group (Figure 1A). Moreover, food intake was improved by increasing P content of the low protein diets. No difference in food intake was observed among the LP-0.1P, LP-0.3P and NP-0.3P groups (Figure 1B, Supplemental Figure 1). The difference in food intake among groups started with the introduction of the various diets and persisted throughout the experimental period (supplementary figure 1). The pattern of variation in energy efficiency [weight gain (g)/100 kcal] was found to parallel that of weight gain, in which energy efficiency of the low protein groups increased with the addition of P to the diet, in which that of the LP-0.015P was lower than that of LP-0.056P that was lower than that of LP-0.1P. However, no differences was observed between energy efficiency of the LP-0.1P and LP-0.3P that was similar to that of NP-0.3P. (Figure 1C).
Body and Liver Composition

Body proximate analysis results suggested that the addition of P to low protein diets was associated with an increase in body moisture. However, the proportion of body moisture was found to decrease with the addition of P to low protein diets. The proportion of moisture in the NP-0.3P group was similar to that of the LP-0.3P group and both were significantly less than moisture proportion of LP-0.015 P and LP-0.056 P fed rats. The addition of P to low protein diets was associated with a significant increase in total body fat content ($P < 0.01$), but no significant changes were observed in the percentage of body fat ($P = 0.23$). Defatted carcass weight was used to provide information on body protein status since mineral content is known to be relatively small. The quantity and percentage of defatted rat carcasses of the LP-0.3P and NP-0.3P treatments were similar to each other regardless of protein content of the diets and both were significantly greater than that of the LP-0.015P and LP-0.056P groups (Table 1).

In the low protein groups, liver weight increased with increasing dietary P but liver weights of LP-0.1 P and LP-0.3P rats were similar to that of NP-0.3P. However, when liver weight was expressed per 100 g of body weight, no statistically significant difference were detected among treatment groups ($P = 0.55$). Additionally, no significant differences were observed among groups in terms of their percentages of hepatic dry weight or fat content. (Table 1).

Plasma results

Total plasma P concentration was significantly different among the groups ($P < 0.01$), in which that of LP-0.015 P treatment had the lowest value, while that of other treatments maintained similar levels. Plasma glucose concentration of the NP-0.3P group was similar to that of the LP-0.3P group. Among the low protein groups, plasma glucose concentration was
found to increase with increased P content of the diet. Plasma TG, total cholesterol, albumin and CRP levels were not found to be significantly different among treatment groups and were not affected by either protein or P content of the diets (Table 2).

In the low protein treatments, plasma urea nitrogen was found to decrease with increased P content of the diet up to 0.1% P. No differences in plasma urea nitrogen were observed among the NP-0.3P, LP-0.1P and LP-0.3P groups (Figure 2).

Liver histology

Analyses of liver photomicrographs (Supplemental Figure 2) indicate that steatosis grade and location, micro vesicular steatosis and portal inflammation were similar among groups and thus were not affected by protein level or P content of the diet. All groups had approximately similar cases of Microvesicular Steatosis. Around 5 to 6 rats from each group presented with Grade 0 or Grade 1 Steatosis, and only 1 rat from each group developed Grade 3 Steatosis. No signs of Mallory Hyaline Bodies, Fibrosis or Glycogenated nuclei were detected in all rats. Only lobular inflammation was found to be significantly greater in the low protein groups as compared to the normal dietary protein group. (Table 3).

Discussion

The study was designed to investigate the impact of dietary P supplementation on several growth parameters of rats maintained on low protein diet. In animals, food intake was reported to be regulated in order to serve two objectives. First, the satisfaction of nutritional needs for growth and maintenance, which entails long-term regulation of intake and this is usually accompanied by an increase in food intake. In support, diet selection and protein restriction studies have shown that young animals are capable of adjusting their intake
in order to support their nutritional protein requirements [29, 30] for growth and maintenance. Second, the maintenance of homeostasis that relates to acute or short-term regulation of food intake [31] and this is usually associated with a decease in food intake in order to avoid the toxicity of amino acid accumulation that is common with the ingestion of low quality proteins. Thus, reduced food intake of the low protein low P diets (LP-0.015P; LP-0.056P) as compared to the other low protein groups (LP-0.1P; LP-0.3P), despite the similarity in dietary protein contents, is likely to have been the outcome of a dietary adaptation for the maintenance of homeostasis [32-34]. Adequate protein metabolism in terms of synthesis and degradation depends on the availability of essential amino acids as well as energy needed to catalyze these reactions. Protein synthesis is an expensive energy-requiring process, 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 [30]. Several observations indicate that this process is highly dependent on P availability [35-37]. In mice, dietary P restriction was reported to lower gastrocnemius muscle tension, due to a slow rate of ATP synthesis, and this was reversed by P supplementation [35]. Additionally, ATP depletion by 2.4-dinitrophenol [36] or fructose infusion [37] was reported to reduce protein synthesis. Hence, under condition of low P intake, the body loses its capacity to synthesize protein due to reduced ATP availability that is dependent on exogenous P replenishment [35, 36] and ultimately amino acids accumulate in circulation. Consequently, food intake is reduced to protect against amino acid toxicity. The ability of P to alter protein metabolism was supported by the fact that dietary P content was inversely related to plasma urea nitrogen. Thus, it can be concluded that the observed increase in food intake with addition of P to low protein diet is likely to have been attributed to an improvement in amino acid homoeostasis due to an enhancement in protein metabolism (e.g. protein synthesis). The facts that proteins are the main sources of dietary P and that bodily protein metabolism is
dependent on P, makes it reasonable to postulate that reduced food intake under low protein diets [38] is highly related to alteration of amino acid homeostasis in a manner which mimics that of low quality protein diet. Accordingly, the reported changes in food intake following protein (casein) manipulation [10] may have been partially related to P content of the diet, especially since 50% of P (0.15% of diet) in rodents diet is derived from casein [24].

On the other hand, measures of body composition seems to be highly dependent on P availability in the diet as indicated by the resemblances in body composition between LP-0.3P and NP-0.3P groups. This is further supported by the findings where differences in body weight between the normal P containing (LP-0.3P and NP-0.3P) and the P depleted (LP-0.015P and LP-0.056P) groups were associated with changes in both body fat and protein. For example, the 10% reduction in water (%) of the NP-0.3P and LP-0.3P groups was almost replaced by 5% increase in fat (%) and 5% increase in protein (%), while the opposite was true for the P depleted groups (LP-0.015P and LP-0.056P). In the latter, the sharp reduction in weight gain was associated with an increase in water (%). Increased water retention is known to be the main feature of Kwashiorkor, which was not explained by protein restriction alone ([10, 39] and is known to be associated with hypophosphatemia [40] and poor dietary P availability [41]. The inability of P restriction to increase hepatic water retention argues against its involvement in the development of kwashiorkor. It is worth noting that body composition changes following P manipulation were mainly attributed to energy efficiency since the magnitude of changes in food intake was modest.

In addition, the high resemblance between LP-0.1P and the control groups (LP-0.3P and NP-0.3P) in weight gain, food intake and body weight was not reflected by similarities in body composition, as the percentage of body protein (defatted percentage) of the 0.3% P (LP-0.3P and NP-0.3P) groups was higher than that of the LP-0.1P group. This further confirms the
importance of P in protein metabolism and indicates that P is capable of altering body
composition. In the present study, no significant alterations in liver composition and
histology were detected despite the fact that dietary P restriction in mice was reported to
increase hepatic lipid accumulation, especially under condition of increased cholesterol
content of the diet [42].

Moreover, the failure of dietary P restriction to significantly affect plasma P content, except
under extreme condition of restriction (LP-0.015P), is in line with human findings [43] in
which plasma P is reported not to be a indicator of P intake. Whatever, dietary P
manipulation (between 0.056%P and 0.3%P) was able to manipulate food intake, energy
efficiency, weight gain and body composition though plasma P was not affected. This further
confirms that plasma P level is neither a good marker of P intake nor an indicator of
changes in food intake or weight measures.

Furthermore, the observed elevated plasma glucose in the control group may have been
related to the effect of egg white protein on insulin secretion [44-46]. Human studies have
shown that insulin incremental area following 50g of egg white ingestion was much lower
than that of cottage cheese in both healthy [45] and diabetic subjects [44]. Additionally, the
ingestion of a breakfast with whole egg, egg white or egg yolk was associated with a rise in
blood glucose concentration following egg white ingestion, as compared to that of whole egg
or egg yolk meals [46]. However, the resemblance in plasma glucose between LP-0.3P and
NP-0.3P implies that plasma glucose is not solely dependent on the quantity of egg white in
the diet. In low protein (egg white) groups, the increase in plasma glucose with the addition
of P possibly indicates that egg white ingestion was able to blunt the effect of P on insulin
sensitivity [47] or the presence of varied glycogen storage between the groups. The latter
option may prevail, since plasma glucose is affected by hepatic glycogen content that is known to be stimulated by P intake [48].

The ability of P intake to manipulate food intake and body composition requires further investigations to determine the level of P intake that is capable of improving body composition.

Low body protein content (defatted %) was present under condition of 0.1%P (LP-0.1P) (about 0.25 mg P/kcal) and lower despite having normal protein intake, while higher levels of P intake improved body protein content. The level of P intake (0.25mg/Kcal of highly bioavailable P) resembles that consumed by most people, which was reported to be about 0.5 mg P/kcal [49] assuming a 50% bioavailability (due to the significant contribution of plant sources). Such level is not thought to enhance protein status. In support, a recent study on humans reported an improvement in body weight and waist circumference of overweight and obese subjects following 12 weeks of P supplementation [50]. Although body composition was not measured, an increase in lean body mass percent would be expected.

Under moderate protein restriction (10%), P addition (0.3%) was able to significantly improve food intake, weight gain and energy efficiency similar to a normal protein diet (20%) containing same level of P (0.3%). Body protein content (defatted %) was high with 0.3% P diet, irrespective of protein content (10 or 20%). Looking at the pattern of changes of the different measures, it seems that dietary phosphorus level of 0.1% may represent a critical threshold. However, it is not clear whether P would be able to exert these effects under added protein restriction. Our results may have implications on the management of malnutrition and may have financial value since the cost of protein is much higher than that of P. Additionally, our findings suggest that not only protein quantity but also P content of the diet is of extreme importance for improving food intake, weight gain and energy efficiency.
Author's Contributions

OO: designed the study, performed the statistical analysis, supervised all the work and had primary responsibility for the final content; RH: collected the data and wrote a draft manuscript; OO and RH analyzed the data; MJ, AT, HG: critically revised the manuscript for important intellectual content. All authors have read and approved the final manuscript.
References


44. Gannon MC, Nuttall FQ, Lane JT, Burmeister LA. Metabolic response to cottage cheese or egg white protein, with or without glucose, in type II diabetic subjects. Metabolism 1992;41(10):1137-45.


Table 1: Body Weight (g) and Body Composition of rats fed a control diet or 1 of 4 low protein diets with different P concentrations for 9 weeks.

<table>
<thead>
<tr>
<th>Variables</th>
<th>LP-0.015P n=9</th>
<th>LP-0.056P n=10</th>
<th>LP-0.1P n=10</th>
<th>LP-0.3P n=10</th>
<th>NP-0.3P n=10</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Body Weight, g</td>
<td>266.1 ± 35.7</td>
<td>265.4 ± 32.8</td>
<td>266.5 ± 27.6</td>
<td>267.8 ± 24.2</td>
<td>266.9 ± 31.9</td>
<td>1.000</td>
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<tr>
<td>Final Body measures</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>383 ± 42.6 a</td>
<td>426 ± 39.5 a</td>
<td>513 ± 50.6 b</td>
<td>536 ± 76.7 b</td>
<td>563 ± 60.6 b</td>
<td>&lt; 0.001</td>
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<td>Water (g)</td>
<td>224.4 ± 25.6 a</td>
<td>237.1 ± 21.4 ac</td>
<td>261.1 ± 22.4 ab</td>
<td>253.5 ± 17.8 bc</td>
<td>277.1 ± 19.5 d</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>(%)</td>
<td>65.4 ± 2.1 a</td>
<td>62.4 ± 3.7 ab</td>
<td>57.2 ± 6.3 bc</td>
<td>53.9 ± 9.7 c</td>
<td>55.1 ± 5.6 c</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>24.9 ± 9.4 a</td>
<td>33.8 ± 15.8 ab</td>
<td>56.6 ± 32.1 bc</td>
<td>64.9 ± 37.7 c</td>
<td>56.4 ± 21.2 ab</td>
<td>0.006</td>
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<tr>
<td>(%)</td>
<td>20.6 ± 5.0</td>
<td>23.1 ± 6.1</td>
<td>27.3 ± 8.6</td>
<td>26.7 ± 8.5</td>
<td>24.2 ± 5.3</td>
<td>0.230</td>
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<td>Defatted (g)</td>
<td>94.5 ± 9.9 a</td>
<td>110.1 ± 14.9 a</td>
<td>142.7 ± 22.5 b</td>
<td>163.2 ± 46.7 bc</td>
<td>174.0 ± 33.7 c</td>
<td>&lt; 0.001</td>
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<tr>
<td>(%)</td>
<td>14.0 ± 3.7 a</td>
<td>14.5 ± 3.9 a</td>
<td>15.5 ± 5.6 ab</td>
<td>19.5 ± 5.6 bc</td>
<td>20.8 ± 5.4 c</td>
<td>0.010</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Weight (g)</td>
<td>12.0 ± 2.1 a</td>
<td>12.5 ± 1.9 a</td>
<td>15.3 ± 2.4 b</td>
<td>16.4 ± 3.3 b</td>
<td>16.3 ± 2.3 b</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Weight (g/100g)</td>
<td>3.3 ± 0.4</td>
<td>3.1 ± 0.3</td>
<td>3.2 ± 0.4</td>
<td>3.2 ± 0.2</td>
<td>3.09 ± 0.2</td>
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<td>Water (%)</td>
<td>72.2 ± 1.5</td>
<td>71.1 ± 2.2</td>
<td>70.5 ± 1.6</td>
<td>70.7 ± 1.3</td>
<td>70.7 ± 1.0</td>
<td>0.187</td>
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<td>Fat (%)</td>
<td>8.0 ± 2.0</td>
<td>13.1 ± 5.9</td>
<td>11.0 ± 3.6</td>
<td>11.9 ± 6.5</td>
<td>11.0 ± 2.7</td>
<td>0.186</td>
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LP-0.015P: 10% protein and 0.015% P; LP-0.056P: 10% protein and 0.056% P; LP-0.1P: 10% protein and 0.1% P; LP-0.3P: 10% protein and 0.3% P; NP-0.3P (Control Group): 20% protein and 0.3% P.

Data are expressed as means ± SD of all values. One-way ANOVA analysis is used to detect significant differences between the groups. Significance is set at P-value < 0.05. Categories in the same row not sharing the same subscripts are significantly different.
Table 2: Plasma Metabolites of rats fed a control diet or 1 of 4 low protein diets with different P concentrations for 9 weeks.

<table>
<thead>
<tr>
<th>Variables</th>
<th>LP-0.015P n=9</th>
<th>LP-0.056P n=10</th>
<th>LP-0.1P n=10</th>
<th>LP-0.3P n=10</th>
<th>NP-0.3P (Control Group) n=10</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphorus, mg/dL</td>
<td>5.4 ± 0.9 a</td>
<td>6.5 ± 1.2 ab</td>
<td>7.4 ± 0.9 b</td>
<td>6.4 ± 0.7 ab</td>
<td>6.4 ± 0.9 ab</td>
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<td>Glucose, mg/dL</td>
<td>148.3 ± 91.7 a</td>
<td>205.0 ± 57.5 ab</td>
<td>245.4 ± 83.3 ab</td>
<td>263.9 ± 68.6 b</td>
<td>277.0 ± 76.5 b</td>
<td>0.004</td>
</tr>
<tr>
<td>Insulin, ng/mL</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.4</td>
<td>0.8 ± 0.8</td>
<td>0.8 ± 0.9</td>
<td>0.7 ± 0.9</td>
<td>0.487</td>
</tr>
<tr>
<td>TG, mg/dL</td>
<td>35.7 ± 6.7</td>
<td>41.2 ± 17.2</td>
<td>45.0 ± 22.7</td>
<td>40.6 ± 15.8</td>
<td>41.2 ± 13.1</td>
<td>0.807</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>87.0 ± 22.1</td>
<td>85.3 ± 13.3</td>
<td>84.8 ± 14.7</td>
<td>85.5 ± 15.9</td>
<td>105.4 ± 25.3</td>
<td>0.081</td>
</tr>
<tr>
<td>Albumin, mg/dL</td>
<td>3.2 ± 0.2</td>
<td>3.2 ± 0.3</td>
<td>3.1 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>3.2 ± 0.3</td>
<td>0.921</td>
</tr>
<tr>
<td>CRP, mg/dL</td>
<td>3.2 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td>2.7 ± 0.7</td>
<td>2.7 ± 0.8</td>
<td>3.1 ± 0.3</td>
<td>0.162</td>
</tr>
</tbody>
</table>

LP-0.015P: 10% protein and 0.015% P; LP-0.056P: 10% protein and 0.056% P; LP-0.1P: 10% protein and 0.1% P; LP-0.3P: 10% protein and 0.3% P; NP-0.3P (Control Group): 20% protein and 0.3% P.

Data are expressed as means ± SD of all values. Significance is set at P-value < 0.05. Categories in the same row not sharing the same subscripts are significantly different.
Table 3: Liver histology results of rats fed a control diet or 1 of 4 low protein diets with different P concentrations for 9 weeks.

<table>
<thead>
<tr>
<th>Variables</th>
<th>LP-0.015P n=9</th>
<th>LP-0.056P n=10</th>
<th>LP-0.1P n=10</th>
<th>LP-0.3P n=10</th>
<th>NP-0.3P n=10</th>
<th>ANOVA P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil-R-O Image Analysis (µm²)</td>
<td>280 ± 727</td>
<td>1510 ± 2837</td>
<td>1234 ± 1636</td>
<td>2075 ± 3088</td>
<td>664 ± 1457</td>
<td>0.407</td>
</tr>
<tr>
<td>Steatosis Grade</td>
<td>0.4 ± 1.0</td>
<td>0.5 ± 1.0</td>
<td>0.9 ± 1.1</td>
<td>0.6 ± 1.0</td>
<td>0.3 ± 0.7</td>
<td>0.701</td>
</tr>
<tr>
<td>Location</td>
<td>0.7 ± 1.0</td>
<td>0.7 ± 1.3</td>
<td>1.8 ± 1.6</td>
<td>0.9 ± 1.5</td>
<td>0.3 ± 0.9</td>
<td>0.118</td>
</tr>
<tr>
<td>Micro vesicular steatosis</td>
<td>0.6 ± 0.5</td>
<td>0.6 ± 0.5</td>
<td>0.9 ± 0.3</td>
<td>0.7 ± 0.5</td>
<td>0.5 ± 0.5</td>
<td>0.385</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>0.7 ± 0.9&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.7 ± 1.0&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4 ± 1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.1 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.020</td>
</tr>
<tr>
<td>Portal inflammation</td>
<td>0.2 ± 0.4</td>
<td>0.2 ± 0.4</td>
<td>0.1 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>0.1 ± 0.3</td>
<td>0.895</td>
</tr>
</tbody>
</table>

LP-0.015P: 10% protein and 0.015% P; LP-0.056P: 10% protein and 0.056% P; LP-0.1P: 10% protein and 0.1% P; LP-0.3P: 10% protein and 0.3% P; NP-0.3P (Control Group): 20% protein and 0.3% P.

Data are expressed as means ± SD of all values. One-way ANOVA analysis is used to detect significant differences between the groups. Significance is set at P-value < 0.05. Categories not sharing in the same row the same subscripts are significantly different.
Figures

Figure 1. Mean daily food intake, weight gain, and energy efficiency of rats fed a control diet or 1 of 4 low protein diets with different P concentrations for 9 weeks. ▲: Control Group: 20% protein and 0.3% of P; ●: Low protein groups (10% protein) with 0.015% P, 0.056% P, 0.1% P or 0.3% P. Food intake, weight gain, and energy efficiency are expressed as Mean ± SD as a function of the level of phosphorus in the diet, n = 9 or 10. Energy efficiency was calculated as the average weight gained per day per 100 kcal of the corresponding diet consumed. One-way analysis of variance (ANOVA), and specific comparisons were made between each of the five groups using Fisher’s pairwise comparisons. Values that do not share the same subscript are significantly different (P-value < 0.05).

Figure 2. Plasma urea nitrogen concentrations (mM) of rats fed a control diet or 1 of 4 low protein diets with different P concentrations for 9 weeks.

▲: Control Group: 20% protein and 0.3% of P; ●: Low protein groups (10% protein) with 0.015% P, 0.056% P, 0.1% P or 0.3% P. The plasma urea nitrogen concentrations are expressed as Mean ± SD as a function of level of phosphorus in the diet, n = 9 or 10. One-way analysis of variance (ANOVA), and specific comparisons were made between each of the five groups using Fisher’s pairwise comparisons. Values that do not share the same subscript are significantly different (P-value < 0.05).
A

Weight Gain (g/day)

0 0.015 0.05 0.1 0.15 0.2 0.25 0.3

Low Protein
Control

B

Food Intake (g/day)

0 0.015 0.05 0.1 0.15 0.2 0.25 0.3

Low Protein
Control

C

Energy Efficiency (g/100kcal)

0 0.015 0.05 0.1 0.15 0.2 0.25 0.3

Low Protein
Control

Dietary Phosphorus (%)
**Supplemental Table 1:** Composition\(^a\) (macro and micronutrients) of the experimental diets (control and 4 low protein diets with different P concentrations).

<table>
<thead>
<tr>
<th>Composition of diet</th>
<th>LP-0.015P</th>
<th>LP-0.056P</th>
<th>LP-0.1P</th>
<th>LP-0.3P</th>
<th>NP-0.3P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg white*, g/100g (% energy)</td>
<td>10 (10)</td>
<td>10 (10)</td>
<td>10 (10)</td>
<td>10 (10)</td>
<td>20 (20)</td>
</tr>
<tr>
<td>Corn Oil, g/100g (% energy)</td>
<td>10 (22)</td>
<td>10 (22)</td>
<td>10 (22)</td>
<td>10 (22)</td>
<td>10 (22)</td>
</tr>
<tr>
<td>Corn Starch, g/100g (% energy)</td>
<td>35 (34)</td>
<td>35 (34)</td>
<td>35 (34)</td>
<td>35 (34)</td>
<td>30 (29)</td>
</tr>
<tr>
<td>Sucrose, g/100g (% energy)</td>
<td>35 (34)</td>
<td>35 (34)</td>
<td>35 (34)</td>
<td>35 (34)</td>
<td>30 (29)</td>
</tr>
<tr>
<td>Cellulose*, g/100g</td>
<td>5.5</td>
<td>5.5</td>
<td>5.4</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Mineral mix (AIN-93G)*, g/100g</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix (AIN-93VX)*, g/100g</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total Energy, kcal/100g</td>
<td>410</td>
<td>410</td>
<td>410</td>
<td>410</td>
<td>410</td>
</tr>
<tr>
<td>KH(_2)PO(_4)*, g/100g</td>
<td>-</td>
<td>0.041</td>
<td>0.091</td>
<td>0.291</td>
<td>0.282</td>
</tr>
<tr>
<td>Total Phosphorus, g/100g</td>
<td>0.015</td>
<td>0.056</td>
<td>0.106</td>
<td>0.306</td>
<td>0.312</td>
</tr>
</tbody>
</table>

\(^a\) As formulated. P: phosphorus.

LP-0.015P: 10% protein and 0.015% P; LP-0.056P: 10% protein and 0.056% P; LP-0.1P: 10% protein and 0.1% P; LP-0.3P: 10% protein and 0.3% P; NP-0.3P (Control diet): 20% protein and 0.3% P.

1 1.5mg P/g Egg White

2 Phosphorous Free Mineral Mix (AIN-93G MIX without phosphorus)

* From Dyets Inc., Bethlehem, Pennsylvania, USA
Supplemental Figure 1. Mean weekly food intake of rats fed a control diet or 1 of 4 low protein diets with different P concentrations.
Online Supporting Material

LP-0.015P: 10% protein and 0.015% P; LP-0.056P: 10% protein and 0.056% P; LP-0.1P: 10% protein and 0.1% P; LP-0.3P: 10% protein and 0.3% P; NP-0.3P (Control diet): 20% protein and 0.3% P.

Statistical analysis using one way analysis of variance (ANOVA) with Fisher’s pair wise comparison shows that weekly food intake of the low protein low P groups (LP-0.015P; LP-0.056P) was constantly lower than that of the other groups.
Supplemental Figure 2: Hepatic histopathological assessment of rats fed a control diet or 1 of 4 low protein diets with different P concentrations for 9 weeks.

Staining and original magnification: Haematoxylin and eosin (H&E) (x 100) and Oil-Red (x 400).