A diet with 35 % of energy from protein leads to kidney damage in female Sprague–Dawley rats

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Abstract

High-protein (HP) diets for weight loss remain popular despite questions surrounding overall safety. In a recent study using the pig model, we showed that long-term intakes from whole proteins at 35 % energy (en %) cause moderate renal histological damage. To examine whether this observation may be species specific or more generalisable, the effect of this diet in rats was examined. Using plant and animal whole proteins, 70-d-old female Sprague–Dawley rats were randomised to either a normal-protein (NP; 15 en %) or a HP (35 en %) diet for 4, 8, 12 and 17 months. Renal function was assessed by creatinine clearance and urinary protein levels, and pathology was assessed by examination of glomerular hypertrophy, glomerulosclerosis and tubulointerstitial fibrosis. Rats consuming the HP diet had 17 % higher kidney weights (P<0·0001), three times higher proteinuria (P<0·0001) and 27 % higher creatinine clearance (P=0·0012) compared with those consuming the NP diet. Consistent with this, HP-fed rats had larger glomeruli (P<0·0001) and more glomerulosclerosis (P=0·0003) compared with NP-fed rats. The HP diet also resulted in altered levels of free monocyte chemoattractant protein-1 (P<0·0001).

The histological changes are consistent with those observed in the pig model. In contrast to the pig model, the elevated proteinuria and creatinine clearance observed in the rat model are also usually observed with HP consumption in human subjects. These results indicate that the rat is a useful model for HP effects on the kidney and, along with previous results using the pig model, suggest that long-term intake of high levels of protein may be detrimental to renal health.

Key words: High-protein diet; Renal hypertrophy; Glomerular damage; Kidney

The European Food Safety Authority(1) is planning to revisit the 1992 European Union dietary protein recommendations and will begin public consultations related to the Dietary Reference Value for proteins this year. In 2002, the Institute of Medicine Committee for the Reference Intakes for Macronutrients set the acceptable intake range for dietary protein in adults from 10 to 35 % of daily energy (en %), to complement the acceptable ranges for fat and carbohydrate(2). However, the Institute of Medicine also stated that there is a lack of published research supporting the safety of long-term high-protein (HP) diets, despite their increasing popularity for use in weight loss and control.

Chronic casein intake in excess of 35 en % results in proteinuria and histological changes in normal and compromised rat kidneys(3,4). When intake of semi-purified protein is near 35 en % throughout the rodent lifespan, a strong correlation between proteinuria and renal lesions is observed(5). The excessive filtration of amino acids as a result of HP diets induces glomerular scarring, and their tubular reabsorption results in inflammation, and ultimately causes fibrosis and tubular cell hypertrophy(6–8). Transforming growth factor β-1 (TGF-β1) and the monocyte chemoattractant protein-1 (MCP-1)/chemokine (C–C motif) receptor 2 (CCR2) axis appear to be involved in the early stages of renal fibrosis, inflammation and hyperproliferation(9–13). These mediators, along with blood homocysteine levels that are often elevated with HP feeding and associated with microalbuminuria(14), may therefore be sensitive markers of early renal damage.

Questions remain, however, regarding the applicability of findings from previous studies of HP diet effects on the
kidney. First, the effects of HP diets were established using primarily casein or other semi-purified protein isolates. Therefore, the effects of HP diets on renal health in which plant and animal whole proteins are substituted with isoenergetic amounts of carbohydrate remain to be determined. Second, the Institute of Medicine discounted evidence from rats because of the belief that the rat kidney is an inappropriate model for the human kidney\(^1\). Therefore, in a recent study, we have provided pigs with whole proteins derived from a mixture of plant and animal sources at the upper end of the range (35 en %) recommended by the Institute of Medicine. After 8 months of feeding, there was evidence of increased kidney size and histological damage, suggesting that long-term intakes of HP diets may compromise renal health\(^{13,14}\). However, the pigs in this study did not display elevated proteinuria with HP feeding, which is in contrast to the findings of increased proteinuria in most HP human studies\(^{14,16,17}\).

Since the vast majority of research on renal physiology and health uses the rodent kidney as the model for investigation, re-examination of HP diets with whole proteins in rodents is warranted and offers a comparison with another mammalian species.

The hypothesis of the present study is that HP intake at 35 en %, which is composed of whole proteins in proportions similar to human consumption, is safe with respect to renal health. The primary objective was to test this hypothesis in normal rats, using HP diets in which the protein was supplied by plant and animal whole proteins, as was done in the previous study using the pig model. The secondary objective was to assess the involvement of TGF-\(\beta_1\), MCP-1, CCR2 and homocysteine in any early renal dysfunction and pathology associated with HP diets.

### Experimental methods

#### Animals and diets

All animal procedures were approved by the University of Manitoba Committee on Animal Care and were in accordance with the guidelines of the Canadian Council on Animal Care. Female Sprague–Dawley rats (Charles River, St-Constant, QC, Canada) at 10 weeks of age were randomly assigned to either a normal-protein (NP, 15 en %) or a HP (35 en %) diet, each formulated to meet the nutrient requirements of rats\(^{18}\) (Table 1). Female rats were chosen to parallel our earlier study in pigs\(^{15}\). The animal:plant protein ratio in the NP diet was 2:1, which is similar to that consumed by humans. In addition, the proportions of animal proteins with respect to meat, poultry and dairy in the NP diet were similar to the proportions of these protein sources in human diets\(^{19}\). Diet ingredients were tested for macronutrient and mineral composition, and the diets were formulated to provide similar amounts of lipid, fibre, Ca, P, K, Na, Mg, Zn, as well as being isoenergetic.

Rats were housed in pairs in solid bottom plastic cages and were provided diets and tap water ad libitum for 4, 8, 12 and 17 months (\(n=8–11\)/diet per time point). During the treatment period, rats were kept in a controlled environment maintained at 21–23°C with 55% relative humidity with a 14 h light–10 h dark cycle. Rats were weighed bi-weekly, and food disappearance while in their normal cages was measured once a week over 3 d. Metabolic cages (Nalgene; Fisher Scientific, Fair Lawn, NJ, USA) were also used to measure 3 d food intakes and to collect 24 h urine samples a week before their respective terminations, with the exception of the rats terminated at 17 months since their size exceeded the capacity of these cages. In these animals, urine samples were collected at termination from the bladder. Therefore, only proteinuria relative to creatinine concentration could be calculated for the 17 months terminations.

At the end of the feeding periods, rats were anaesthetised with a mixture of ketamine–xylazine and exsanguinated via cardiac puncture to obtain blood samples, which were placed immediately on ice and allowed to clot before centrifuging to obtain serum for analysis. Left kidneys were removed, weighed and sectioned in half longitudinally across the hilum, with one half fixed in 10% buffered formalin for histological analyses. The other half was snap-frozen in liquid N\(_2\) and stored at –80°C for analyses of TGF-\(\beta_1\), MCP-1 and CCR2.

<table>
<thead>
<tr>
<th>Ingredient</th>
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<th>HP</th>
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<tr>
<td>Formulation (g/kg diet)</td>
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<tr>
<td>Wheat (14·6 % protein)</td>
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<tr>
<td>barley (10·8 % protein)</td>
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<tr>
<td>Low-ash poultry meal (67·4 % protein)*</td>
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<td>Pork meal (57·4 % protein)*</td>
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<tr>
<td>Egg albumen (74·6 % protein)†</td>
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<td>Skimmed milk powder (34·1 % protein)‡</td>
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<tr>
<td>Mineral mix (AIN-93M without Ca and P)§</td>
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<td>Na</td>
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<tr>
<td>Zn (parts per million)</td>
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<td>49·9</td>
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AIN, American Institute of Nutrition.

* Rothsay, Winnipeg, MB, Canada.
† Inovatech, Abbotsford, BC, Canada.
‡ Dairyland, Red Deer, AB, Canada.
§ Harlan Teklad, Madison, WI, USA.
¶ Added to the diets to balance Na, P and K levels.
** Added to balance the lactose content of the HP diet.
†† More lard was added to the NP diet because the added protein sources in the HP diet contributed small amounts of extra fat to the HP diet.
Renal and serum biochemistry

Serum and urinary creatinine were measured using the Jaffe reaction as modified by Heinegård & Tiderstrom (20), and adapted for microassay. Urinary and kidney protein contents were measured using the Bradford method for total protein (21). Serum total homocysteine and cysteine were analysed by reverse-phase HPLC (22, 23). Concentrations were determined based on external standard curves with inter- and intra-assay CV < 2%.

Renal histology and image analysis

The formalin-fixed left kidney was embedded in paraffin, sectioned at 5 μm and processed using our published methods (24, 25). Transverse tissue sections were stained with periodic acid Schiff for glomerular diameter measurement. Those for quantitative analysis of glomerulosclerosis and tubulointerstitial fibrosis were stained with Sirius red (in adaptation of Masson's trichrome stain), which permits image analysis measurement using a standard incandescent microscope light source.

Images were captured with a Spot Junior charge-coupled device camera by random stage movement throughout the renal cortex and were analysed with Image Pro version 6.0 (Media Cybernetics, Silver Spring, MD, USA). An average of twenty-five images per kidney was collected for all histomorphometrical analyses, and all measurements were carried out in a blinded fashion. Glomerulosclerosis and tubulointerstitial fibrosis were measured by densitometry, and glomerular volume was determined with standard stereological techniques developed by Weibel (26), as described previously (24).

Transforming growth factor β-1, monocyte chemoattractant protein-1 and chemokine (C-C motif) receptor 2 analyses

Frozen left kidneys were lyophilised, pulverised and mixed, and a representative sample was homogenised in 100 volumes of ice-cold particulate homogenisation buffer, centrifuged at 100,000 g for 30 min at 4°C, and the supernatant was stored at −80°C until further analysis. The homogenate buffer was identical to the particulate buffer as described previously (27), except that the level of Triton X-100 was 0.5%. Commercial rat ELISA kits were used for the determination of free MCP-1 (Biosource International, Inc., Camarillo, CA, USA) and TGF-β1 (R&D Systems, Inc., Minneapolis, MN, USA) in duplicate according to the manufacturer’s instructions. The total amount of free MCP-1 and TGF-β1 present in the kidney tissue is expressed per mg of renal protein, which was determined by the Bradford method (21).

Western immunoblotting of CCR2 on the renal homogenates was performed as described previously (28, 29). Detection of CCR2 was carried out by incubating blots overnight at 4°C with a CCR2 antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation for 1 h at room temperature with a peroxidase-conjugated secondary antibody (1:20,000; Sigma, St Louis, MO, USA and Oakville, Ontario, Canada).
ON, Canada). Immunoblots were incubated with ChemiGlow (Fisher Scientific, Nepean, ON, Canada), and image analysis and quantification of immunoreactive bands were performed using the Fluorchem Q digital imaging system (Alpha Innotech, San Leandro, CA, USA). A reference control was applied in duplicate to each gel so that results could be compared across gels. Dose–response curves were used to determine the linear range of response (14 μg of protein were used).

Statistical analysis
Data were analysed using a general linear model 2 X 4 ANOVA with diet and time as factors using SAS software (SAS Institute, Cary, NC, USA). Normality of the data was tested using a plot of actual v. predicted residuals and the Shapiro–Wilk’s W statistic on the residuals. Non-normal data were transformed if possible. The MIXED procedure was used when data could not be normalised (feed disappearance and kidney weight relative to body-weight data). Outliers were excluded if the plotted residuals exceeded the pooled standard errors of the means X 3. Effects were considered significant at P<0.05.

Results
Body weight and fat mass
Rats that consumed the HP diet had body weights that were 8% lower compared with rats that consumed the NP diet, despite no differences between dietary treatments in feed intake based on disappearance data (Table 2). However, feed intake data derived from metabolic cages suggest an 8% reduced intake in HP-fed rats (P=0.0584, data not shown). The effects of these diets on body composition and bone properties in the present study have been published in detail, and in sum, the HP diet lowered body fat content without hindering the mechanical and weight-bearing properties of bone (30). These body composition data reveal that the lower body weights could be attributed to 24% lower body fat in rats given the HP diet compared with NP-fed rats (30).

Renal and glomerular hypertrophy and glomerulosclerosis
Kidney weights and protein content were 18 and 17% higher, respectively, in rats from the HP and NP diet groups (Table 2). This difference in kidney size was observed whether expressed as kidney mass by itself or relative to body weight (Table 2) or lean body weight (data not shown). Glomerular hypertrophy (Fig. 1(a) and (b)) and glomerulosclerosis (Fig. 1(c) and (d)) were evident, with glomerular size being 22% and glomerulosclerosis 33% higher in the kidneys of HP-fed rats compared with those of NP-fed rats (Table 3). No effect of diet on tubulointerstitial fibrosis was observed between the groups, but as with the other histological variables, it increased with time (Table 3).

Renal function
Filtration rate as estimated by creatinine clearance was significantly elevated in rats given the HP diet (Table 3). Consistent with the histological alterations in glomeruli in rats given the HP diet, proteinuria was markedly elevated in rats given the HP diet whether expressed relative to creatinine clearance (Table 3) or on a 24 h basis (data not shown).

Effect of high-protein diets on renal and plasma biomarkers
Animals offered the HP diet had 16% lower free MCP-1 than NP-fed rats, with no differences in renal CCR2 or TGF-β1 between the diets. Serum cysteine levels were 7% lower in HP-fed rats, while homocysteine levels were not different between the diets (Table 4).

Fig. 1. Representative images of glomeruli from rats given the (a) normal-protein (NP) or (b) high-protein (HP) diet for 4 months, stained with periodic acid Schiff, 20x magnification. Arrows point to individual glomeruli. Glomeruli from rats given the (c) NP or (d) HP diet for 17 months, stained with Sirius red, 40x magnification.
Discussion

Long-term feeding of an HP diet using animal and plant whole proteins at 35
eN % resulted in renal hypertrophy, hyperfiltration and proteinuria, along with enlarged and more sclerotic glomeruli. These differences were moderate, but consistent with previous studies in rats that examined renal function and histological changes with high levels of purified protein sources (3–5, 31–33). It is also consistent with reports of rats from several toxicological studies with diets composed primarily of plant proteins in which renal pathology was worse in rats consuming diets with 27
v.
16 en % for up to 2 years (34–36). However, the HP diet in these studies also had lower fat and higher fibre content causing the rats to consume more than the control rats, which is unusual with HP feeding, and may have contributed to the effects on kidney. Finally, it is also consistent with our findings of renal and glomerular hypertrophy and histological changes in pigs given similar diets with animal and plant whole proteins (15). The consistency of these findings indicates that whether dietary proteins are given in whole mixed form or singly in a more purified manner, HP diets have small, but significant, effects on the normal kidney, which may compromise renal health.

The results of the present study also indicate that the rat is an appropriate model for studying dietary HP effects on renal pathology and function. The observed effects of dietary HP on renal size, filtration and proteinuria in our rats are consistent with what has been observed in human studies. In human subjects, HP intakes for up to 3 months result in higher filtration rates (37–39), and HP intakes over 6 months increase kidney volumes (40). In addition, increased proteinuria is usually exhibited in individuals given HP diets over the long term (14, 16, 17), although this is not always observed (40). Similar to the human model and present findings in the rat model, dietary HP caused renal hypertrophy in our previous study using the pig model. However, in contrast to studies in pigs, the effects of dietary HP on renal function in our rat model were not significant, although the observed renal function in our rat model was increased only in the short term and urinary protein excretion was not increased in pigs. On the other hand, urinary protein excretion was increased in pigs, while the urinary protein levels in the present study did not exhibit increased urinary protein in rats. This discrepancy of the HP effects on homocysteine between the results of pig and rat models, and the variability in the human studies, suggests that elevated homocysteine is not a necessary component of the HP effects on renal histology.

The histological effects are more difficult to compare with human studies, as studies on the effects of dietary HP in healthy human subjects have not been performed. However, meta-analyses of human studies conclude that decreasing protein intake in the presence of renal disease is advantageous in delaying a decline in renal function (44–46), as is the case in studies with rats (31–33). Since histological changes occur long before changes in renal function, it is very likely that improved histological parameters would accompany the improvements in renal function. This would be consistent with the improvements in renal histological parameters that were observed in the present study in rats given the HP diet.
observed in our studies in both rat and pig models. Hence, there are characteristics of each model, which reflect the renal response to dietary HP in human subjects, but the effects in rats compared with pigs appear to resemble the human response, at least under the conditions of the present study. Although, clearly, more studies under a variety of different conditions need to be undertaken before conclusions regarding the appropriateness of specific animal models can be made, these studies do indicate that data from the rat or pig model should not be discounted.

Since there was evidence for adverse, albeit moderate, effects of the HP diet on the normal kidney in the present study and the previous study in pigs, those with mildly compromised kidneys may be at greater risk for detrimental effects of HP diets. Although strictly not 'normal', this population has relevance to the dietary recommendations for healthy individuals because significant proportions of the adult population consider themselves to be normal, despite having mild renal impairment (stage 1 or 2 chronic kidney disease), of which they are unaware (47, 48). Those with mild renal impairment in the Nurses' Health Study in the highest quintile compared with the lowest quintile of dietary protein intake had a greater decline in glomerular filtration rate over a 10-year follow-up (49). Furthermore, higher body weight increases chronic kidney disease risk (50, 51), and obese individuals commonly have mild renal impairment (52, 53), even when corrected for increased body surface area. Consistent with this, in overweight individuals, increased protein consumption was associated with increased microalbuminuria, which continued to rise as daily protein intake increased (14). Therefore, renal health of overweight and obese individuals who are interested in HP diets for weight loss should be examined before this strategy is deployed to avoid further possible deterioration in renal function. This is important because although the rats in the present study displayed renal damage despite loss of body fat, the overall benefits of weight loss need to be assessed within the overall effects on health.

Kidneys from rats given the HP diet had lower levels of free MCP-1 than those given the NP diet. We have observed that in a rat model of renal disease, free renal MCP-1 levels are markedly reduced in diseased rats compared with normal rats, while CCR2 levels are significantly elevated (54). This suggests that when CCR2 is elevated and binds to MCP-1, the amount of free MCP-1 is reduced. Hence, the reduced level of free MCP-1 may be an early marker of activation of the MCP-1–CCR2 axis, but this remains to be further investigated.

The American Diabetes Association recommends a low-carbohydrate or low-fat diet as an option for weight loss (55). This recommendation comes with a caution that protein intake should not exceed 20% and that HP diets be avoided altogether in those with diabetes. These caveats should be heeded until more is known regarding the long-term effects of HP in populations at risk for and with early renal disease. Although there was evidence for adverse effects of HP diets on kidney function in rats compared with pigs, the present study is not conclusive in assessing the appropriateness of specific animal models to predict the human response. At least under the conditions of the present study, HP diets in rats appear to resemble human renal responses in both rat and pig models. Hence, we observed in our studies in both rat and pig models, which reflect the renal response to dietary HP in human subjects, but the effects in rats compared with pigs appear to resemble the human response, at least under the conditions of the present study.
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References