Urinary Protein Excretion in Mice Consuming High Casein and Soy Diet: Importance of Sex and Protein Source

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Abstract
High protein intake can accelerate kidney disease. Soy protein diet has been reported to reduce the progression of chronic kidney disease (CKD) compared to a milk (casein) protein diet. Sex differences play a key role in kidney function and disease with premenopausal women having fewer kidney diseases compared to age-matched men. The purpose of this study was to compare the effects of high soy protein consumption with that of high casein consumption on renal protein excretion in male and female mice. We hypothesized that sex differences exist in the renal handling of these two protein sources.

Two separate studies, one with male mice and one with female mice, were conducted. Mice at 25 days of age were placed in metabolic cages. After a baseline period of three days on standard diet, one group consumed 40% casein and the other an isocaloric 40% soy diet for 25 days (n=6 per group). Daily measurements included body weight, food and water intake, urinary flow rate (UFR), and urinary protein excretion (UPE) via semi-quantitative dipstick analysis. UPE (mg/day) was also determined on the final day of each study by measuring total urine protein concentration (TPC) and calculating UPE (UPE = TPC x UFR).

Independent of the diet, females excreted significantly less protein compared to males despite equal protein consumption. Regardless of sex, the casein groups excreted more protein than the soy groups via dipstick measurement. UPE (mg/day) measured on the 25th day was higher in the casein groups compared to the soy groups (males: 31.9 ± 2.5 vs. 17.8 ± 0.9, $p<0.009$; females: 10.2 ± 1.7 vs. 5.4 ± 0.8, NS).

We conclude that high soy intake induces less proteinuria compared to that with high casein intake. Female mice on high casein and high soy diets excreted less protein when compared to male mice on the same diet. These results support the benefits of soy protein in patients with kidney disease and indicate sex differences in the renal handling of high protein diets.

Introduction
The rising incidence of obesity is a global health concern. A sedentary lifestyle coupled with an unhealthy diet contribute significantly to this public health problem.[1] Adjustment from the unhealthy high fat diet to the healthy high protein diet can produce positive benefits. Indeed, high protein diets offer a possible strategy to reduce obesity as studies have shown that such diets can be effective in weight reduction.[2-4] In humans and animals, the effects of a high protein diet depend on the composition and quantity of dietary protein.[5,6]

Animal and plant proteins constitute the two primary sources of dietary proteins and reports have shown that the former increases hypercholesterolemia whereas the latter does not.[7] Nevala and

colleagues demonstrated that a casein-based protein diet accentuated the development of hypertension in the spontaneously hypertensive rat (SHR) in comparison to the SHR fed a soy-based diet.[8] Isoflavones such as genistein, glycitin, and daidzein in the soy diet appear to be involved in lowering blood pressure and cholesterol, thus lowering the risk for cardiovascular disease.[7,9] Isoflavones also lower blood pressure via the reduction in oxidative stress[10], as well as via reductions in angiotensin-converting enzyme activity.[11] The US Food and Drug Administration (FDA) in October 1999, approved the labeling of foods containing soy protein as being protective against coronary heart disease.[9] In 2000, the American Heart Association (AHA) Nutrition Committee released a scientific advisory on soy protein and cardiovascular diseases which supported the cardio-protective effects of soy protein.

Interestingly, various effects of protein intake on kidney function have been studied for many years with evidence showing that both low and high protein intake can adversely affect kidney function.[12,13] Obesity and hypertension predispose people to renal disease and while high protein consumption might benefit the obesity condition, it can potentially harm the kidney. Despite the cardio-protective effects of soy protein, the overall effect of high protein intake on renal function is still debated. High protein consumption enhances the workload of the kidney and while it may not be of major concern with the healthy individual, it can cause adverse effects in humans with kidney disease.[3,14,15] Park and colleagues demonstrated that soy-based diet delays the progression of chronic kidney disease compared to other protein sources and also showed beneficial effects in patients with end-stage renal disease.[10]

Premenopausal women are protected against renal disease compared to age-matched men and although the mechanisms responsible for this protection remain unknown, the sex steroids appear to play major roles.[16] Carneiro and colleagues reported that female sex hormones prevent the development of hypercholesterolemia-induced renal dysfunction and decrease urinary protein excretion (UPE), a key indicator of renal function, in mice.[17] Hence, the sex as well as the source of dietary protein may have differential effects on the level of renal damage and UPE under conditions of high protein intake.

Given the worldwide rise in obesity and hypertension and the impact of sex on renal disease, it is important to investigate the effects of high protein intake, protein source, and the role of sex on renal function. The present study was conducted to compare UPE values in male and female mice consuming a high protein diet of either casein or soy. Here we present evidence demonstrating that protein source and sex both matter in UPE from high protein consumption.

Methods

Animals: Intact male and female CD-1 mice were purchased from Envigo (Indianapolis, IN). Mice arrived to OSU-CHS and were housed in a temperature- and humidity-controlled room with automatic 12-hour off-and-on lighting in the institution's animal facility. Mice were placed in plastic bins with *ad libitum* access to standard diet and water before starting the experiment. The OSU-CHS IACUC approved all experimental procedures with animals.

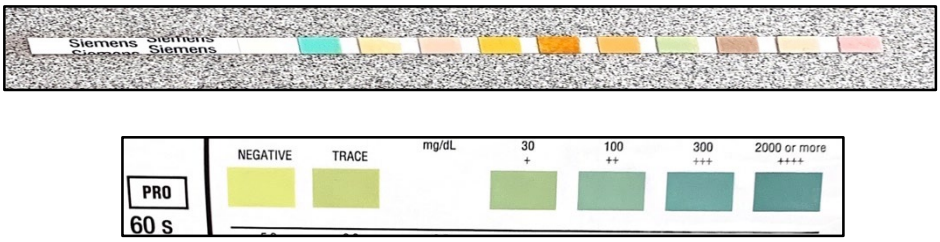
Procedures: The study included two experimental protocols – one with males and one with females – and each protocol consisted of two groups (n = 6/group). At 25 days of age, mice were placed in metabolic cages and consumed a standard diet of 20% casein for the first three

days. Mice were then given the high protein diet with one group consuming 40% casein and the other 40% soy for 25 days. The protocol was thus divided into two periods: 1) baseline period for the first three days; and, 2) the experimental period for the next 25 days.

Daily measurements included body weight, food and water consumption, urine volume, urine osmolarity, and UPE. Food and water intake were determined by weighing the respective containers at the beginning and end of the 24-hour period. Urine volume was determined by weighing the urine container during the same 24-hr period and used to determine the urinary flow rate (UFR) in ml/day.

Strip UPE Measurement: The key measurement in this study was the semi-quantitative values of UPE which we measured daily via urinalysis strips (Siemens Multistix®, Tarrytown, NY).

Urinalysis Strip with protein indicator (fourth marker from right)



UPE of each mouse was measured on the numerical scale from 0 to 4 based on the strip above where Trace = 0, 30 = 1, 100 = 2, 300 = 3, and 2000 or more = 4. Measurements were taken in blind manner in that the person reading the strip did not know which mouse urine was measured.

Hospital UPE Measurement: On the final day, we sent urine samples to the OSU-CHS Medical Center laboratory for analysis of urine protein and creatinine concentrations via the colorimetric method with pyrogallol red and molybdenum acid using an Olympus AU400™ analyzer.

High Protein Diets: Diets were obtained from Envigo Teklad. The composition of each diet is given in the table below.

Table 1. Composition of the high casein and high soy diets.

Constituent	**40% Casein (g/Kg)**	**40% Soy (g/Kg)**
Casein	460.0	
Soy		460.0
Sucrose	231.82	232.92
Corn Starch	200.0	200.0
Corn Oil	50.0	50.0
Cellulose	15.0	15.0
Mineral Mix	13.37	13.37
Calcium Phosphate		16.0
Calcium Carbonate	17.4	2.7
Vitamin mix	10.0	10.0
Ethoxyquin, antioxidant	0.01	0.01

Statistics: Data are shown as mean ± se and were analyzed using GraphPad Prism 6 software (Carlsbad, CA). A repeated measures 2-way ANOVA over time was used to determine differences in body weight, food (g) and water (ml) consumption, urine volume and osmolarity, and UPE. ANOVA was used to determine differences in UPE from the urine samples analyzed on the final day of the study. Tukey's test was used to carry out multiple comparisons. A p-value < 0.05 was considered statistically significant.

Results

No differences in any measurements including body weight, food intake, water intake, UFR, UPE, were measured during the three-day baseline period.

Body Weight: Figure 1 shows that the body weight profiles of both male and female mice demonstrated normal growth curves for young healthy mice. There was no statistical difference between the casein vs soy groups in either sex; although, the male casein group was slightly, though non-significantly, higher than the male soy group.

Figure 1

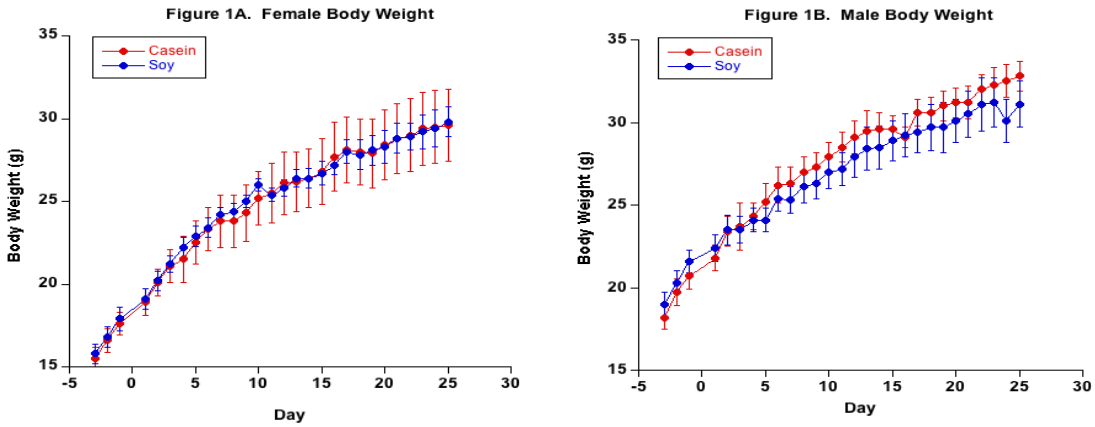


Figure 1. Daily body weight (mean ± se) of female (1A) and male (1B) mice on casein and soy diet. There was no difference in body weight between the casein and soy groups in the female or male study.

Food and Water Intake: No differences in food intake (g/day) were measured with respect to either protein source or sex while on casein and soy: Males: 5.15 ± 0.06 vs. 4.99 ± 0.09, respectively; and Females: 4.88 ± 0.08 vs. 5.03 ± 0.07, respectively. Likewise, no differences in water intake (ml/day) were measured with respect to either protein source or sex while on casein and soy: Males: 8.89 ± 0.18 vs. 8.62 ± 0.31, respectively; and Females: 8.47 ± 0.18 ml vs. 8.64 ± 0.21, respectively.

UFR: UFR (ml/day) did not differ based on the protein source (casein vs soy) in males (5.13 ± 0.15 vs. 4.99 ± 0.09, respectively) or females (5.73 ± 0.15 vs. 5.32 ± 0.15, respectively). However, UFR in females was significantly higher than males on the respective diet ($p < 0.05$).

Figure 2

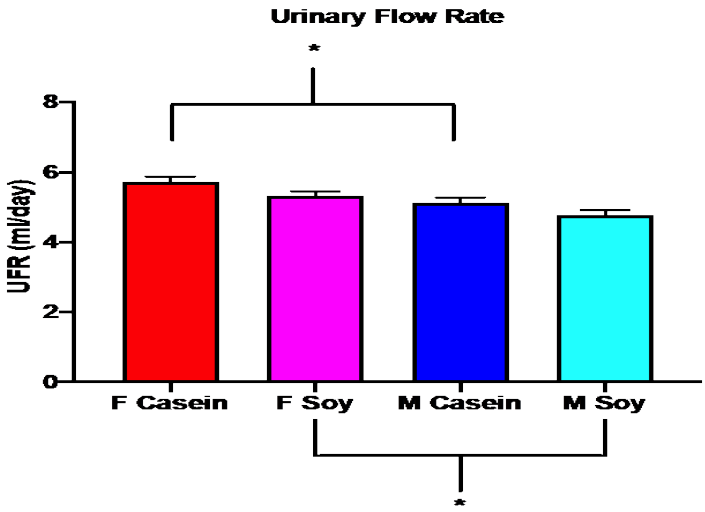


Fig 2. Average daily UFR (ml/day) measured as reported in Methods. (* - $p < 0.05$)

Dipstick UPE: Figure 3 shows average UPE from daily semi-quantitative measurements via urinalysis strips or dipsticks. Dipstick values from casein diets were significantly higher than those from soy diets in both males and females. Males on casein diet had significantly higher values than females on casein diet (1.95 ± 0.08 vs 1.50 ± 0.08, respectively, $p<0.01$). Males on soy diet had higher, non-significant values than females on soy (1.26 ± 0.06 vs 1.10 ± 0.01, respectively).

Figure 3A

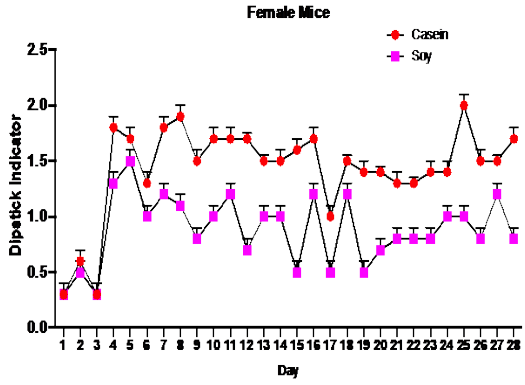


Figure 3B

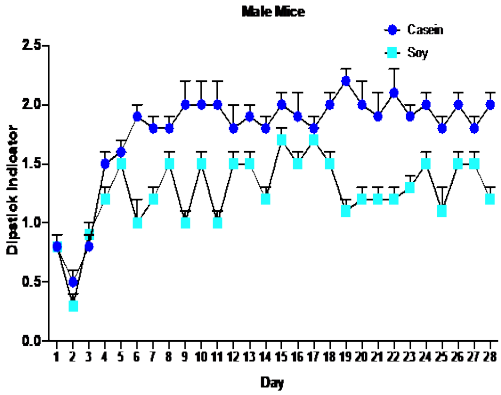


Figure 3C

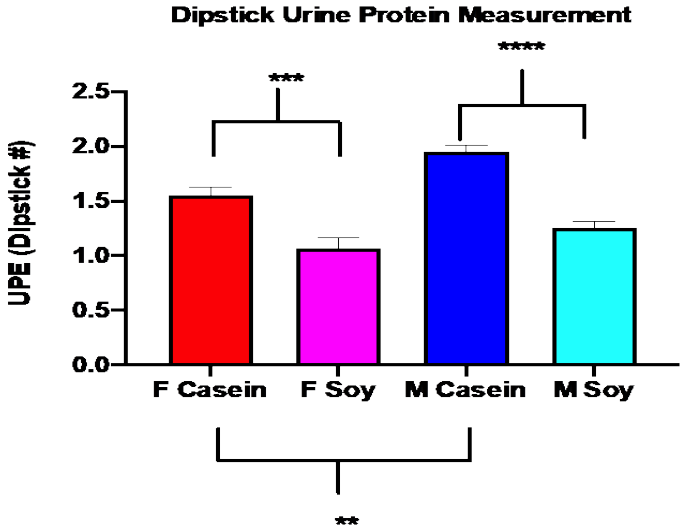


Figure 3. A & B: Daily dipstick measurement of female and male measurement. C: Average daily UPE as determined via dipstick analysis. Analysis technique reported in Methods. (**** $p<0.0001$; *** $p<0.001$, ** $p<0.01$)

Hospital UPE Measurement: On the final day of each study urine samples were delivered to the OSU-CHS Medical facility and analyzed for total protein concentration (TPC, mg/ml). UPE (mg/day) was calculated for each mouse (TPC x UFR). Figure 4 confirms the dipstick measurements in that male casein group had higher UPE than the male soy group (31.9 ± 2.5 vs 17.8 ± 0.9, respectively, $p<0.0001$). UPE for the female casein group was higher (but not significant) than the female soy group (10.2 ± 1.7 vs 5.4 ± 0.8, respectively).

Figure 4

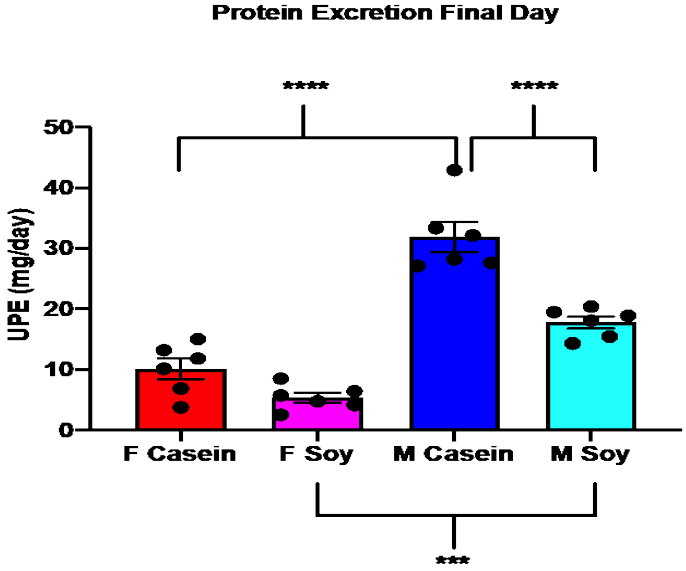


Figure 4. Protein excretion measured on the final day of the study. The urinary protein concentration of each mouse was measured, and protein excretion was determined based on the UFR on that day. (**** - $p<0.0001$, *** - $p<0.001$)

Urine Protein-to-Creatinine ratio (PCR) on Final Day: To further establish the sex and protein-source differences, UPE was normalized to urinary creatinine excretion. Urine creatinine was measured at the OSU Medical Facility via a colorimetric technique. Figure 5 confirms that the casein groups had higher UPE compared to the soy groups and that males excrete higher protein levels than females.

Figure 5

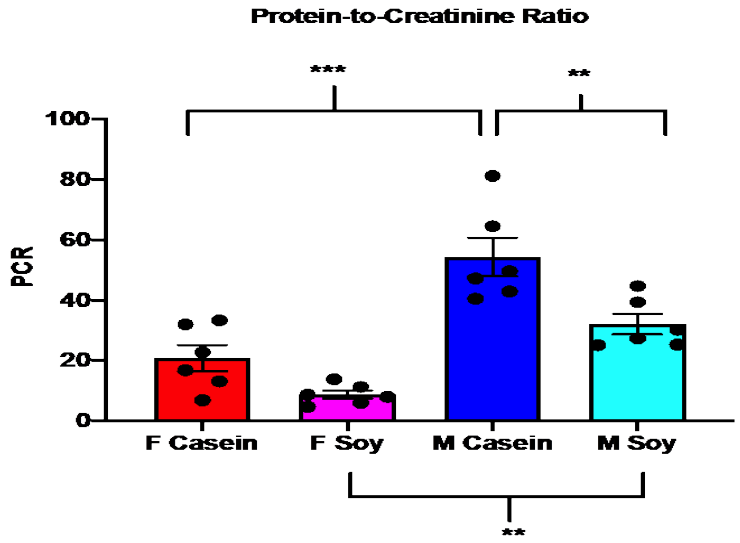


Figure 5. PCR measured on the final day of the study. (*** $p<0.001$; ** $p<0.01$)

Kidney Weight-to-Body Weight: There was no difference in kidney weight-to-body weight (kw/bw, mg/g) ratio between the casein vs. soy diets in either male or female mice. Overall, male kw/bw ratio was significantly greater than that for female mice (0.017± 0.001 vs. 0.013± 0.001, respectively, $p<0.01$).

Discussion

The potentially detrimental effects of high protein intake on the kidney with regard to hypertrophy and enhanced renal blood flow were first shown in the 1920s[18,19]. When one considers the physiology of dietary metabolism, it is not surprising that protein intake, compared to that of carbohydrate and fat, can significantly impact kidney function. The body stores excess carbohydrate and fat in the liver, adipose tissue, and muscle. However, excess protein intake undergoes hepatic breakdown and the resulting metabolites, particularly amino acids and urea, are delivered to the kidney for excretion. This increases renal blood flow, glomerular filtration rate (GFR), and the overall workload on the kidney.[20,21] Indeed, low protein diets are often prescribed for patients with kidney disease. However, it is also important to note that low protein intake approaching malnutrition reduces GFR and renal blood flow[13].

Today's popularity of high protein diets is due to their benefits on weight reduction and preventing obesity.[4] Numerous studies have shown that the source of protein matters and the soy-based proteins have received great attention with regard to cardiovascular and renal function.[6,22-24] Teixeira et al. reported that soy protein delays the progression of diabetic nephropathy.[25] In a recent review, Rafieian-Kopaei et al. concluded that soy is a beneficial protein for preventing or managing chronic kidney disease.[26] In a thorough review of the evidence on soy protein and renoprotection, McGraw et al. stressed that even though an established relationship appears to exist between the benefits of soy protein and kidney function, further studies are needed.[27]

Sex differences in cardiovascular and renal function are well known and it is widely accepted that premenopausal women are protected against these diseases compared to age-matched males[16]. Given our interest in sex differences in renal function, the purpose of this study was to compare the effects of high casein and high soy protein intake on UPE in young, healthy male and female mice. While male mice have been shown to yield higher UPE values under normal conditions, it is important to compare different protein sources in both sexes consuming high protein diets in order to advance the base of knowledge on sex differences and in treating obesity.[28] We hypothesized that high casein consumption would yield higher UPE than that from high soy intake in both sexes and that female mice would demonstrate lower UPE levels.

Young, healthy male and female mice consumed isocaloric diets of high soy and high casein for a period of 25 consecutive days after a three-day baseline period. Body weight profiles demonstrated normal growth patterns for this age and strain (Web Search "Growth Curve" from www.envigo.com for ICR-CD1® mice). Mice consumed the same amount of food and water throughout the study and no abnormalities in any animal were noted. We suspected the protein source might result in a difference in the kidney weight-to-body weight (kw/bw) ratio, but this did not happen. It is certainly possible that a longer consumption period with casein could result in higher kw/bw compared to that of soy. As expected, kw/bw ratio in males was significantly greater than that for females. This observation is well known and due to the anabolic effect of testosterone.[29]

Female mice on casein and soy protein diet had slightly though significantly higher UFR compared to males on those respective diets (Fig. 2). These results could provide explanations for the cardio-renal protection in females. It is reasonable to propose that the higher UFR in females plays an important role in blood pressure regulation. With regard to the present study, one would expect that with higher UFR, higher UPE would be observed. However, this was not the case. The combination of higher UFR in females and higher UPE in males emphasizes the sex difference in UPE shown in this study.

The key results of this study clearly indicate that the protein source and sex both matter with regard to UPE from high protein consumption. High casein consumption yielded higher UPE compared to that of high soy consumption and females had significantly lower UPE. The semi-quantitative dipstick measurements (Fig. 3) were confirmed by the quantitative measurements of UPE (Fig 4) on the final day of each study. Further confirmation comes from the UPC measurements (Fig. 5) showing that males had higher UPE when corrected for creatinine excretion which allows for adjustment of lean body mass. Casein has been reported to be a

better source of protein compared to plant proteins due to its richness in all the essential amino acids.[24,30] However, the effect of casein on UPE is of significant concern particularly for those with renal disease. Soy protein, a phytoestrogen, has an estrogenic effect that is believed to be protective against kidney disease.[31] Thus, these results support using soy over casein for those concerned with renal disease.

The sex difference shown in this study may be attributed to the effects of sex steroids on the kidney. Doublier and colleagues[32] reported that testosterone induced apoptosis of cultured podocytes. They also demonstrated that estradiol protected podocytes from the androgen-induced pathological effects. Baylis[33] and Neugarten[34] suggested that androgens in male rats cause glomerulosclerosis and proteinuria. Verzola and colleagues reported that testosterone causes kidney hypertrophy and can disrupt the structure of the glomerular basement membrane.[35] Blush and colleagues reported that estradiol reduces TGF-β-induced collagen synthesis and renal injury in mice.[36] Maric and colleagues reported that supplementation of estrogen in ovariectomized Dahl salt-sensitive rats reduces glomerulonephritis and tubule interstitial fibrosis.[37] Controversy exists as to which steroid plays the key role in renal function and disease; protection via estrogen or pathology via testosterone. We suspect that both steroids play important roles in these sex differences and studies with gonadectomized mice supplemented with the sex steroids or placebo will shed important light in this regard.

In summary, evidence from this study clearly showed that mice consuming a high protein diet of the casein source have significantly higher UPE compared to mice consuming a high protein diet of the soy source. Additionally, male mice have significantly higher UPE compared to female mice consuming the same amount of protein. We conclude that both the protein source and sex matter with regard to UPE under high protein consumption. Patients suffering from kidney disease require low protein diets to reduce disease progression, but low protein consumption can cause malnutrition. This study can help guide those in clinical practice to prescribe the proper dietary source for those patients.

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