Original Research Article

The Effect of High Casein Diet on the Histology and Function of Rat Kidney

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ABSTRACT

The obesity epidemic is on the rise and due to the current obesity problem; effective weight loss treatment is of importance and recent studies have shown that a short term treatment with increased dietary protein intake and reduced carbohydrate is more effective but there are concerns of the effects of high protein intake on kidney health, especially in the elderly population. Different proteins affect the progression of renal damage both in the renal ablation models and in human renal diseases differently.
Till date very few studies have been done to study the role of high protein intake in the development of renal damage in rat models. This study was designed to assess the effects of high protein intake on the renal function and histological structure of the kidney in male Wistar Albino rats. The animals on high protein diet had significantly higher levels of urea in serum and urine, uric acid, creatinine, calcium, alkaline phosphatase, total protein and albumin compared to the control group. Histological and electron microscopic examinations of rat kidneys revealed degenerated epithelial nuclei with extensive degeneration of mitochondria. These results suggest that a high protein diet for even a short period of time can cause significant degeneration of epithelial nuclei and serious alteration in the levels of serum and urine biochemical parameters and normal renal histology.
Keywords: High protein diet, Kidney function, renal damage.

INTRODUCTION

Chronic kidney disease remains an important public health threat globally; secondary to the incidence of diabetes and hypertension, also the most common cause of kidney disease. [1] While it is certainly important that we include a balanced intake of protein in our diet, too much protein could have adverse health effects. Previous studies have shown that the quality of protein present in diet affects the progression of renal damage both in the renal ablation models and in human renal diseases [2, 3] which could partially explain the sudden increase in the global incidence of end stage renal disease (ESRD) over the last 3 decades worldwide. [4] The growing proportion of the elderly population also accounts for the total increase in the number of patients admitted for renal replacement

therapy (RRT) with a dramatically high morbidity and mortality rate. [5]

Low protein, low fat and high carbohydrate diet used to be recommended for weight management before but recent studies showed that short term dietary treatment with increased protein and reduced carbohydrate intake is a more effective treatment for weight loss. [6,9] Many studies have shown positive effects on blood lipids, inflammation, blood pressure and other parameters [6,7,10] depending on type of protein ingested, for example, long term soy protein consumption decreases nonadipose tissue lipotoxicity while a high casein diet showed an opposite effect.[6] On the other hand, it is known that the increase in the intake of protein in diet regardless of type, significantly increases urea output in urine and its concentration in plasma and the hypertrophy of kidney. [11] Secondly, a high protein intake itself can cause kidney damage and increased albumin excretion. [12,13]

In animal models, mammals fed on acute and chronic high protein diets are associated with an increase in glomerular filtration rate (GFR) and renal blood flow. [14] These changes, which are comparable to those observed in humans, led to the hypothesis that high protein intakes are associated with progressive glomerulo-sclerosis in the rat. Even in cats fed on a higher protein diet, kidneys showed significant renal. [15] However, these lesions could have been the result of either increased protein or calorie intake in this group of cats. Another study showed that in young rats, high protein intake reversibly increases GFR out of proportion to body weight and stimulates kidney growth by stimulating cell proliferation. [16]

Studies documenting high protein intake as a cause of renal disease in any animal model have not been done. Rather, studies have typically focused on the interaction between protein intake and renal function in the diseased state. Here, we examined the histological changes in kidney by electron microscopy and studied changes in the level of biochemical parameters of rat kidney in response to consumption of 50% high protein diet (casein).

MATERIAL AND METHODS
Animals and diets

Thirty two male Wistar Albino weaning rats, 4-6 weeks old were obtained from the animal care center of King Saud University with an average weight ranging from 70.5-93.5 grams. Animals were randomly divided into 2 groups, control and experimental group (16 rats/group). The rats were housed on a 12/12-hr-light-dark cycle. The control group was given a normal chow diet while the experimental group was given a high casein diet, a total of 17 kilograms normal chow diet and one of 50% casein diet was mixed with water and then made into balls and dried in the oven +35°c then fed to experimental rats group. The protein concentration of experimental diet was adjusted according to the purity of the protein (casein 90.6%). Rats had free access to food and water for the length of the study (6 weeks). The composition of the experimental diets is presented in Table 1. Body weights and food intake were measured weekly. Rats were caged as pairs in metabolic cages and urine was collected every 24 hr for the measurement of urinary biochemical parameters such as urinary creatinine, calcium, urea and uric acid. Immediately after 12 h of food deprivation, 16 rats in each dietary group were weighed and sacrificed by decapitation after anesthetization with CO_2. Blood was collected by heart puncture and was centrifuged at 1,000 g for 10 min. Serum was separated and stored at -20°C for further analysis. The liver and kidneys were removed, weighed and then were processed

for light and electron microscopic studies. Institutional guidelines for animal care and use were followed. The Institutional Animal Care and Research Advisory Committee of King Saud University approved the animal protocol.

Histopathological study

For light microscopy, kidneys were fixed in 10% paraformaldehyde, dehydrated in an ascending grade of ethyl alcohol, cleared in xylene and embedded in paraffin wax which were sectioned at micrometer sizes (μm), and stained using hematoxylin/eosin (H/E). A computer-assisted color image analyzer (light microscope Olympus CX31) was employed to study randomly selected glomeruli from each animal at400x magnification. Glomerular size was measured in tissue sections of 5 μm thick stained with H/E. The outline of 20 glomeruli from each animal was digitized from the light microscope using a video camera and a computer-based analysis system (light microscope Olympus CX31).Renal tissue sections were displayed on the computer screen, and their area was measured by an interactive procedure with an image-analysis software package. Photographs of the desired results were obtained using digital research photographic microscope at 200x and 400x magnification.

For electron microscopic study, fragments of kidney, 2mm in diameter, were selected from the cortex and medulla and fixed in 2% glutraldehyde, then immersed in 1% osmic acid for an hour at room temperature and finally dehydrated and embedded in resin. Thin sections were cut with glass knives using ultra microtome. Sections were placed on copper grids, stained by lead citrate and uranyl acetate and examined in electron microscope at the King Faisal Specialist Hospital and Research Center, Riyadh, Kingdom of Saudi Arabia.

Biochemical analysis of blood and urine

The blood samples were collected by heart puncture from each animal in separate tubes for the analysis of serum levels of blood urea nitrogen, uric acid, creatinine, total protein, calcium, albumin, and alkaline phosphatase. The blood samples were centrifuged at 3,000 rpm (Beckman centrifuge, England) for 10 min. Serum was separated and kept at -70°c for further analysis. An analysis of serum and urine samples was done using an automated chemistry analyzer (Dade Behring, US). Cartridges were brought for each parameter measured.

Statistical Analysis

Statistical analysis was done using SPSS (Statistical Package for Social Sciences). All variables measured were expressed as means ± SE. All variables were normally distributed hence no log transformation was done. Independent t-test was done for comparison of control and experimental rat mean values. Correlation analysis was also done to determine associations of different variables of interest. Differences were considered significant at $P \leq 0.05$.

RESULTS
Study sample characteristics

The baseline weight of both control and experimental rats were comparable (p-value 0.99) (Table 1). Both groups had a significant increase in weight on the sacrificing day, but when compared, animals in the control group significantly gained more weight than those in experimental group (p = 0.0005). The protein intake by the experimental group was almost double of that consumed by the control group (317.34±20.88, 174.95±12.21 respectively). When the physical characteristics of the liver and kidney was compared, it was observed that the experimental group had an

enlarged liver with significantly more weight (0.320 gm. liver wt. /100gm body wt.) when compared to the control group (0.22 gm liver wt. /100gm body wt.).The weight of both right and left kidneys among control and experimental group showed no statistically significant difference while the average weight of the kidney per 100gm body wt. showed that the excess intake of protein in the experimental group resulted in significantly heavier kidney weight ($p<0.001$) than observed for the normal protein intake in the control group (0.074gm kidney wt. /100gm body wt., 0.058gm kidney wt. /100gm body wt.) respectively.

Table 1.Mean±SD of weight, diet consumed, liver and kidney weights and their average weight/100gm body weight for both control and experimental rats at post-test period.

Character	N	Control Group	Experimental Group	T-Value	P-value
Baseline weight (g)	16	83.98 ± 8.47	83.95 ± 6.01	0.012	0.99
Final weight (g)	16	281.79 ± 26.37	239.11 ± 33.02	4.04	0.0005*
Right kidney weight(g)	16	1.19 ± 0.16	1.20 ± 0.19	-0.208	0.84
Left kidney weight(g)	16	1.25 ± 0.14	1.27 ± 0.22	-0.323	0.75
Liver weight(g)	16	8.85 ± 1.11	10.02 ± 1.38	-2.653	0.01*
Total Average Diet consumed (g)	8	760.72 ± 53.15	634.63 ± 41.77	5.28	0.000*
Total Average protein consumed(g)	8	174.95±12.21	317.34± 20.88	-16.65	0.000*
Average diet consumed/100 gm body wt	8	19.55 ± 0.46	20.51 ± 0.88	2.74	0.016*
Average Kidney wt/100 gm body wt	8	0.058 ± 0.005	0.074 ± 0.009	4.48	0.001*
Average liver wt/100 gm body wt	8	0.221 ± 0.009	0.320 ± 0.034	7.93	0.000*

* Significant at $P<0.05$,N=Number of rats, wt= weight

Serum Biochemical markers

The serum urea in the experimental group animals was observed to be significantly higher than those in the control group by almost 3 folds (Table 2).

The levels of Serumuric acid, calcium, total protein as well as serum alkaline phosphatase were found to be significantly higher in the experimental group ($p = 0.000$) when compared to the control group. Other parameters which include serum creatinine and serum albumin were significantly higher in the experimental rats ($p=0.001$) as well, when compared to the control group.

With respect to reference values (Boehm et al, 2007), the mean values of control rats were almost within the acceptable range with the exception of creatinine and uric acid levels which were lower as well as total protein concentration which was elevated with respect to the normal range. In the experimental group, only serum urea, calcium, alkaline phosphatase total protein and albumin were observed to be higher compared to reference values.

Urinary biochemical parameters

The urine of experimental group had significantly higher levels of urea, uric acid, creatinine and calcium when compared to the control group (p-values ≤ 0.05)(Table 3).This may justify the presence of higher concentration of total proteins and hence elevated protein metabolism and burden on experimental animal's kidneys.

Table 2:Mean+SD of serum biochemical parameters for control and experimental rats post-test period

Parameter	Control (N = 16)	Experimental (N = 16)	T-Value	P-value	Normal Values
Urea (mmol/L)	5.29 ± 0.91	14.11 ± 2.25	-14.53	0.000*	7 (4.0-9.3)
Uric acid (µmo/L)	0.15 ± 0.11	0.42 ± 0.79	-7.97	0.000*	1.77(1.003-3.186)
Creatinine (µmol/L)	26.06 ± 3.93	31.50 ± 4.31	-3.74	0.001*	37 (31-48)
Calcium (mmol/L)	2.31 ± 0.18	2.61 ± 0.13	-5.17	0.000*	2.3 (2.02-2.48)
Total protein (g/dl)	6.17 ± 0.31	6.77 ± 0.45	-4.41	0.000*	5 (4-6)
Albumin (g/dl)	4.01 ± 0.25	4.30 ± 0.16	-3.89	0.001*	3.99 (3.71-4.27)
Alkaline phosphatase (IU/L)	156.25+22.5	204.13+34.13	-4.68	0.000*	132 (65-193)

* Significant at p ≤ 0.05, N= Number of rats, Normal values taken from Boehm et al, 2007.

Table 3: Mean+SD of total excretion of urinary biochemicalparameters of control and experimental rats per six weeks at Post-test period

Parameter	Control Group	Experimental Group	T-Value	P-value
Urea mmol	32792.3 ± 15805.9	109718.7 ± 64555.4	2.84	0.018*
Uric acid µmol	35690.0 ± 10996.3	137790.0 ± 31041.3	7.59	0.000*
Creatinine µmol	531123.3 ± 372568.9	3549096.7 ± 1977428.9	3.67	0.004*
Calcium mmol	214.2+83.42	1079.4 ± 862.24	2.45	0.034*

* Significant at p ≤ 0.05

Urinary excretion pattern of different biochemical parameters

Figure 1a indicates the urinary excretion pattern of control and experimental groups during the 6 weeks experiment period. A sharp increase was observed in the urinary output among the experimental group during the second and third weeks, eventually reaching a plateau in the subsequent weeks and modestly declining during the last week. The control group on the other hand, had a more stable urine output with a modest increase between the third and fourth week before achieving a steady output in the last three weeks.

The urinary urea levels show an increasing pattern in the experimental group as compared to the control group all over the experiment period (Figure 1b). It increases remarkably in the 3rd week followed by a sharp decline from week 3 to week 4 while during the subsequent weeks the levels were almost comparable in the experimental and control groups. The control group, on the other hand showed an increase in urinary urea levels in the 1st week and almost comparable levels in the subsequent weeks.

The most noticeable observation was the urinary uric acid levels being higher in the experimental group as compared to the control group throughout the experiment period with remarkable increase during week 4 (Figure 1c). This difference was noted even at 1st week, which was highly suggestive of the influence of high dietary protein intake in the experimental rats.

Urinary creatinine levels were found to be higher in the experimental group as compared to the control group from week 1 onwards and showed a high increase in the 5th week (Figure 1d). A sharp increase in the urinary calcium levels in the experimental group during the 2nd week was also observed with the levels becoming stable in the 3rd week followed by a decline in the 4th week, eventually reaching a plateau in the subsequent weeks. The control group, on the other hand, had more stable urinary calcium levels with a modest increase between the 1st and 2nd week (Figure 1e).
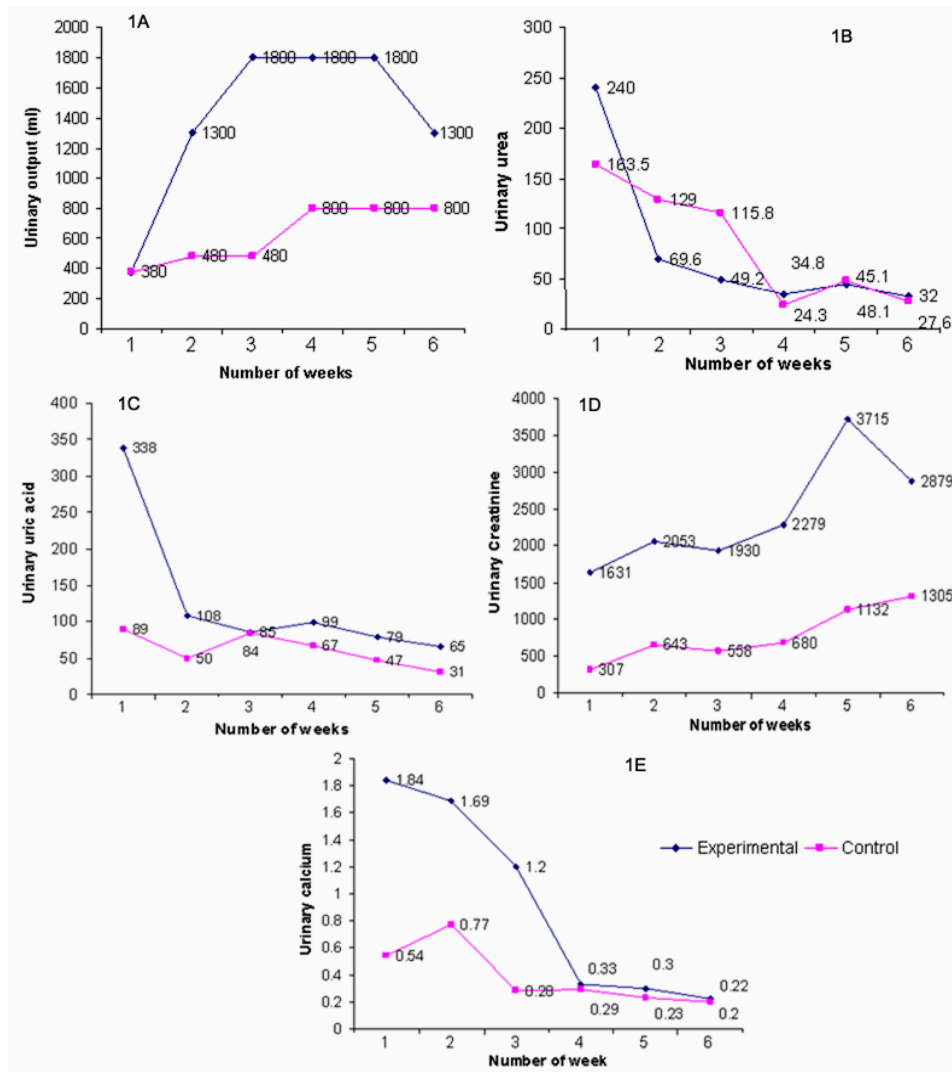
Figure 1. Urinary excretion patterns of 1a.Urine volume; 1b.Urea; 1c.Uric acid; 1d.Creatinine; 1e.Calcium, in control and experimental groups over the 6 week period.

Histopathological Study

Microscopic examination of control kidney showed a normal appearance of both cortexes with normal renal corpuscle. They appeared as dense rounded structures with the glomerulus surrounded by a narrow renal or Bowman's spaces. On the other hand, kidneys of rats administered with high casein diet revealed both cortical and medullary changes such as shrinkage of the

glomerulus in 56.25% of rats leaving wide Bowman's space in 50%of experimental animals. Partial thickening of the capsule and presence of tubular casts in both cortical and medullary tubules, ducts and loop of Henle were seen in 62.5% rats in addition to focal tubular epithelial cells degenerations (Fig. 2).
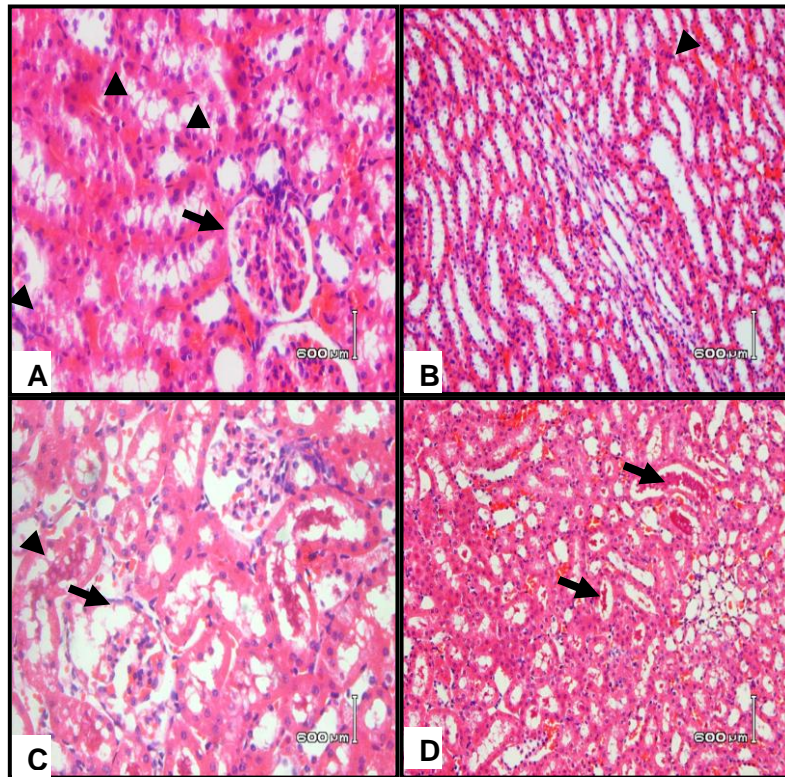

Figure. 2: Light photomicrographs of rat renal tissue. (A&B) represent cortex and medulla of control rat with normal renal corpuscles (arrows) and cortical tubular epithelium (arrow-heads). (C&D) represent renal cortex and medulla of rats received high dose of animal protein showing focal thickening of renal capsule (arrow), shrinkage of the glomeruli (stars), casts in the lumen of cortical tubules (arrow-head) in addition to degeneration of the cytoplasm of the cortical tubular epithelium (stars). Also the medulla shows tubular casts (arrows) (H&E stain. A&C X400 while B&D X 200)

Electron microscopic study of control kidney showed normal details about both cortex and medulla. Epithelial cells of proximal tubules also showed their characteristic microvilli, large nucleus, and basal distribution of large number of longitudinally arranged mitochondria. Few sections of medulla showed normal epithelial cells of collecting tubules those had no microvillus, normal cells of loop of Henle and normal interstitial cells (Fig.3a). However, experimental group showed cortical changes in the form of widening of pedicles of podocytes, focal fusion of pedicles and focal increase of the mesangium. Most of the epithelial cells of proximal and distal tubules had degenerated cytoplasm and nucleus, ballooned mitochondria with irregular cristae. The medullary tubular epithelial cells especially that of collecting tubules showed degeneration of epithelial nuclei along with the mitochondria (Fig.3b).
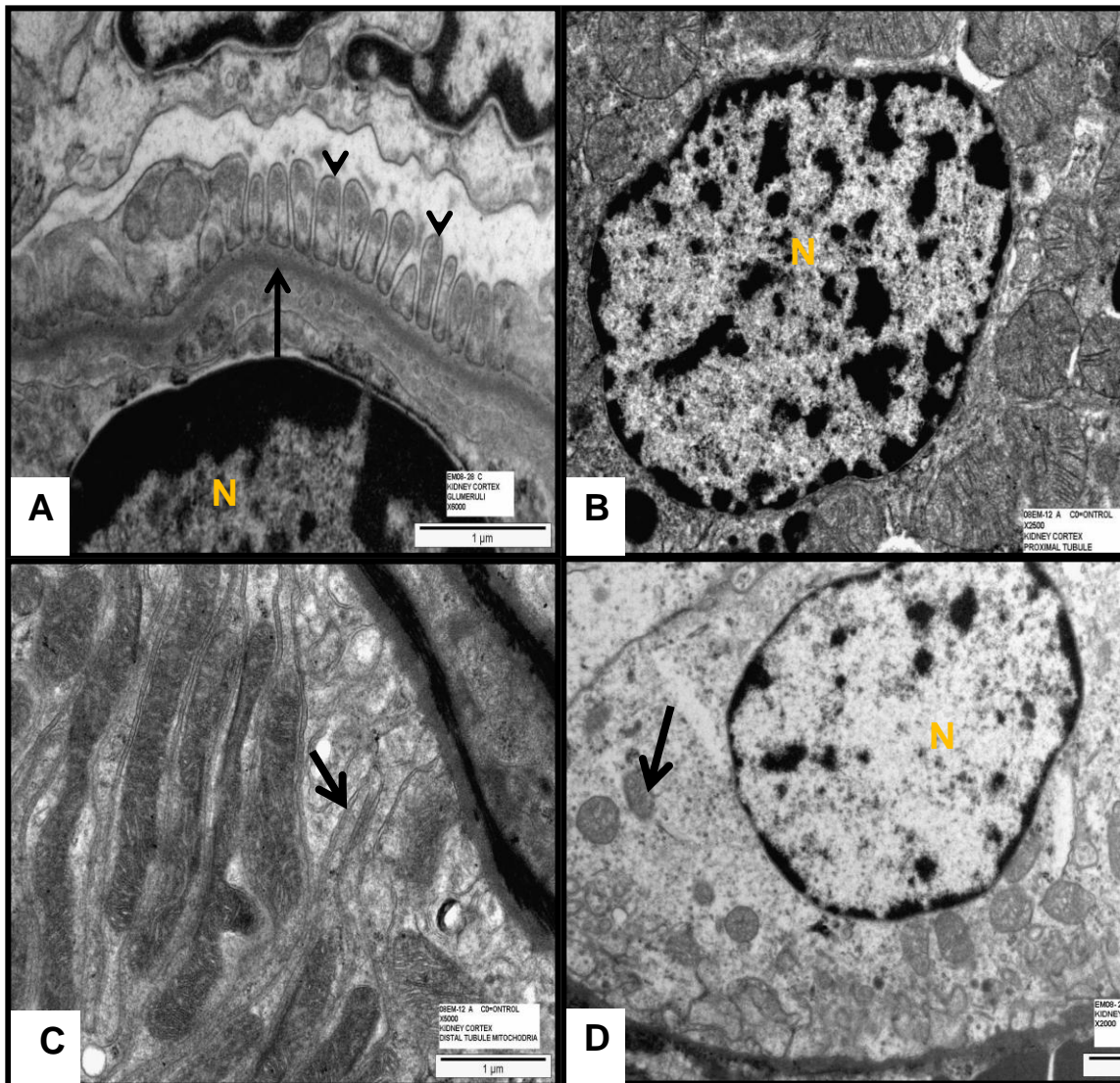
Figure 3a: Electron micrographs of normal control kidney show, (A, B & C are of renal cortex). A, part of renal corpuscle with normal thickness of basement membrane (arrow) , Pedicles of podocytes (arrow-heads) and nucleus (N) of glomerular endothelial cell. B, shows a part of an epithelial cell of proximal convoluted tubule with normal oval centrally located nucleus (N), that surrounded by large number of healthy mitochondria (M). C, represent the basal region of an epithelial cell of distal tubule with basally arranged elongated mitochondria (M) within basal folds (arrow). D, shows characteristic epithelial cell of medullary collecting tubule that has central pale rounded nucleus (N) that surrounded by cytoplasm that contains few small-sized mitochondria (arrow).
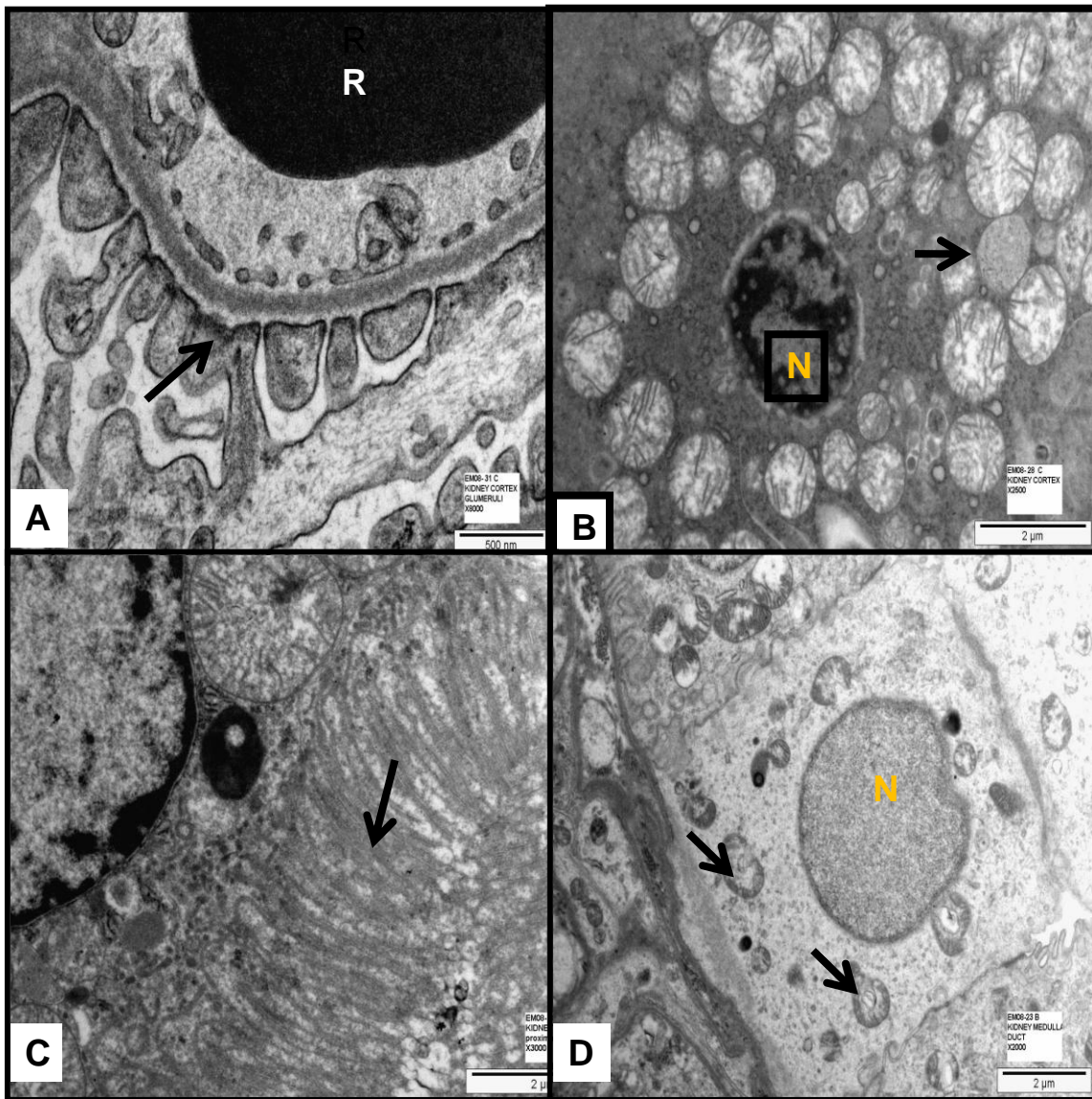
Figure 3b: Electron micrographs of experimental rat kidneys showing (A,B&C of renal cortex). A, shows part of glomerular capillary containing an electron-dense of RBC and fenestrae of an endothelial cell (arrow-heads). Pedicles of podocytes are slightly widened (star) and few of them are fused (arrow). B, represent an epithelial cell of proximal tubule with abnormally small dark nucleus (N) and many swollen mitochondria with irregular cristae (arrows).C, shows microvilli of epithelial cell of proximal tubule (arrow) with degenerated cytoplasm (star). D, represent an epithelial cell of medullary collecting tubule with degenerated nucleus (N) and vacuolated mitochondria (arrows).

DISCUSSION

The high casein diet in experimental models has been shown previously to elevate levels of serum urea, uric acid, creatinine, calcium, total protein, albumin and alkaline phosphatase. [16-18] Our results found a significant increase in the levels of these parameters as well, which could be an indicator of early renal damage in response to consumption of high casein diet. Our study also demonstrates that the high protein diet is directly linked with the elevated protein metabolism and hence, burden on experimental animal's kidneys. Furthermore, the light microscopic study of experimental rats' kidneys showed both

cortical and medullary changes in the form of shrinkage of the glomerulus that leave a wide Bowman's space¸ partial thickening of the capsule presence of tubular casts in both cortical and medullary tubules and ducts¸ in addition to focal tubular epithelial cells degenerations, as reported earlier. [17] In addition, electron microscopic sections of experimental group showed epithelial cells of proximal and distal tubules that had degenerated cytoplasm, nucleus and ballooned mitochondria with irregular cristaeas compared to the kidney of control rats. As expected, the liver weights of experimental animals were heavier as compared with the control group since urea is an excretory end-product of protein metabolism in mammals, making its levels directly proportional to protein intake; and the formation of urea takes place in the liver. This explains why the liver organs in the experimental rats were hypertrophied resulting in heavier mass as reported previously by Braod et al. [18]

We also observed an increased 24-hour urine output in the experimental rats, which could be due to the direct correlation of protein intake to GFR, which in turn increases urine output as observed by Lacroix et al.; [14] and Shaw and Patience. [19] It is known that dietary protein has an effect at the level of GFR in the kidneys. GFR has also been shown to increase by 20–30%within 1 hour of consuming a high-protein meal and that if this were to stimulate urine output; it could affect blood pressure by reducing blood volume and increasing viscosity. [20] De Castro et al (1992) showed that, among 20-80 yr-old men and women, protein intake positively correlated with total fluid intake. However, this apparent relation did not remain when other dietary factors (e.g. carbohydrate, fat and sodium) were considered with multivariate analyses, and the researchers concluded that the primary determinant of fluid ingestion was the amount of solid food ingested, not due to specific macronutrient. [21]

In this study, urinary urea, uric acid, creatinine and calcium levels were significantly higher in the experimental as compared with the control rats. A diet rich in protein is associated with high urinary uric acid excretion and low urinary pH. [22] Uric acid solubility decreases dramatically at a urinary pH lower than 5.5, leading to uric acid crystal formation. [22,23] With regards to calcium, recent studies demonstrate that an increase in urinary calcium is mainly associated with a high consumption of sodium and protein rather than consumption of dietary calcium. [24] Consequently, a decrease in the consumption of sodium and protein is recommended for reducing urinary calcium levels. [25] Despite the elevations in the urinary analyses, it was noted in the figures of urinary urea, uric acid, creatinine and calcium that the elevations were transient and that in the long run the same values were almost comparable to control rats. These changes in the urinary biochemical indicators could be regarded as physiologic and not pathologic which was postulated by Fouque and Aparicio (2007); Lentine (2004) and Jakobsson et al. (2008). Knight et al (2003) in their recent observation stated that high protein intake was not associated with renal function decline in women with normal renal function. However, high total protein intake may accelerate renal function decline in women with mild renal insufficiency. [16,26-28]

In conclusion, a high protein intake in Wistar rats fed for 6 weeks showed changes in the serum and urine levels of markers of renal function which indicate abnormalities in the function of the kidney compounded by changes in the histological structure of the kidneys.

This study acknowledges the limitations that glomerular filtration rates were not measured which could provide more conclusive results. The difficulty was encountered in the urinary collection of rats and that's the reason why individual urinary collection was not achieved. Data presented here, therefore, were based on the descriptive difference as presented in figures and not statistically by comparison of means. Kidney damage might have been more visible if the length of time frame of the study was increased and the intake was sustained. The increased food intake rate increases the animals' use of energy pathways for maintaining the body and coping with the increase in metabolic output pathways such as urea metabolism and metabolic waste excretion. A body (human or animal) easily accommodates high intake of food easily, [29] but as observed, it comes at a cost of increase in organ size (liver and kidney as seen during this study) and histopathological distortion of organelles within the kidney.

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