Impact of high cholesterol diet in mediating inflammation provoked calcinosis in renal tissue of experimental rats

Rajeswari R, Divya J, Jayasudha E, Thellamudhu G, Suresh M, Thulasi Raman K, Prema V & Kalaiselvi P*
Department of Medical Biochemistry, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani campus, Taramani, Chennai-600 113, India

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Several scientific studies suggest the detrimental effects of dietary cholesterol in augmenting renal calcification. However, the scenario behind age associated hypercholesterolemia and renal stone formation is still a mystery. Thus the endeavor of this research was to highlight the impact of high cholesterol diet (HCD) in urolithiasis during aging. Male albino rats of Wistar strain (3 months-young and 24 months old-aged) were used in this study. Hypercholesterolemia was induced by the diet comprising of the normal rat chow supplemented with 4% cholesterol and 1% cholic acid. Determination of urinary stone forming risk factors, membrane damage markers, expression of few lithogenic and anti-lithogenic proteins and renal histopathology were analyzed. The results revealed the elevated concentration of urinary stone forming factors such as uric acid, creatinine, oxalate, calcium, and phosphorus levels, whereas citrate level was decreased in aged hypercholesterolemic rats. Increased risk of stone formation was dictated by the increased expression of albumin and decreased expression of anti-lithogenic Tamm-Horsfall glycoprotein (THP) in the renal tissue of young and aged hyperlipidemic rats. Moreover, increased activities of urinary marker enzymes and brush border membrane enzymes in aged HCD fed rats substantiate renal tubular damage. Histopathological studies confirmed the accretion of lipid and heightening of renal inflammation. These observations validate the role of hypercholesterolemia pertaining to lithiasis during aging. Our analysis also depicts that advanced age is not only risk factor for the formation of kidney stones, but its coexistence with other risk factors such as hypercholesterolemia will make the kidneys more sensitive to renal calculi formation.

Keywords: Aging, Brush border, Membrane enzymes, Hypercholesterolemia, Renal stones, Tamm-Horsfall glycoprotein, Urine, Biochemistry

Kidneys are the organs primarily affected by age even in the absence of other age-related diseases. Renal aging, characterized by loss of function and increased vulnerability to other disorders, are mainly due to increased glomerulosclerosis, tubular atrophy and interstitial fibrosis. Aging may also provoke various metabolic and biochemical abnormalities associated with the nephritic syndrome, focal and segmental glomerular sclerosis. Numerous renal pathologies aggravated by senescence are associated with alterations in cholesterol metabolism. Several studies have demonstrated that hypercholesterolemia alone can aggravate ischemic and nephro toxic acute renal failure in rats. Experimentally, hypercholesterolemia has been shown to decrease renal blood flow, glomerular filtration rate and ultrafiltration co-efficient, expediting glomerular and tubular injury.

There are evidences that glomerular injuries observed during hypercholesterolemia are due to direct interaction of low density lipoprotein (LDL) and oxidized LDL with mesangial cells. Moreover, hypercholesterolemia was found to cause heightening of inflammation and increased renal oxidative stress. Thus, obesity coupled with senescence may oblige additional burden, exacerbating the anomalies on kidney, deteriorating their function and thereby limiting general health and life expectancy. Further, obesity was also established to be related with excretion of calcium oxalate stone forming risk factors.

Epidemiologically, obesity is linked to nephrocalcinosis and altered urine composition, strongly influencing the pathophysiology of stone formation. The most common crystalline constituents of human urinary stones are calcium phosphate and calcium oxalate. About 80% of gallstones are composed of cholesterol. Though the metabolic and biochemical alterations (sensitive indicators of renal injury) have been well documented in aging and

*Correspondence:
Phone: +91 9841371726; Fax: +91 44 24540709
E-mail: pkalaiselvi2011@gmail.com
hypercholesterolemic rats, scanty information is available on the role of hypercholesterolemia as a urolithic risk factor during aging. Hence the picture of certain renal proteins along with the urinary biochemical alterations will dictate age associated hypercholesterolemia acts as a co-conspirator of lithogenic threat or not. The present study evaluates the lithogenic menace posed by the combinatorial stress of aging and hypercholesterolemia, on the basis of salient biochemical indices and urinary chemistry.

Materials and methods

Chemicals

Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade and were obtained from Sisco Research Laboratory (Mumbai, India). Polyvinylidene Fluoride (PVDF) membrane was purchased from Millipore (Billerica, MA). Primary antibody (Ab) for Tamm-Horsfall glycoprotein (THP) was purchased from Santa Cruz Biotechnology, Inc., Albumin and Immunoglobulin G Kappa (IgG κ) were obtained as kind gifts from Dr. Santhosh kumar, Assistant Professor, USA. Luminol reagent was purchased from Pierce (Rockford, IL). Horseradish peroxidase (HRP) conjugated goat anti-rabbit Immunoglobulin G (IgG) and rabbit anti-mouse Abs were purchased from Bangalore GENEI (India). Methanol and all other chemicals were purchased from Sisco Research Laboratories Pvt Ltd. (India).

Animals

Male albino rats of Wistar strain (Rattus norvegicus) (3 months and 24 months old) were used in this study. Animals were obtained from Central animal house facility, Taramani campus, University of Madras and maintained as per national guidelines and protocols, approved by the Institutional Animal Ethical Committee (IAEC No. 01/014/10). The animals were housed two per cage in large spacious cages under conditions of controlled temperature (25 ± 2 °C) with 12/12 h light/dark cycle and were given food and water ad libitum.

Hypercholesterolemia was induced by the high-cholesterol diet (HCD) comprising of the normal rat chow (feed contained 5% fat, 21% protein, 55% nitrogen free extract, 4% fiber (wt/wt) with adequate mineral and vitamin contents) supplemented with 4% cholesterol and 1% cholic acid15. They were divided into four groups of six rats each, and the grouping of animals is as follows: Group I: Control young rats were fed with normal rat chow and received vehicle (0.89% NaCl) alone; Group II: Young rats were fed with HCD for 45 days and received vehicle alone; Group III: Control aged rats were fed with normal rat chow and received vehicle alone; Group IV: Aged rats were fed with HCD for 45 days and received vehicle alone.

Rats were pre-acclimatized in the metabolic cage for 2 days before urine collection, and they were given sufficient food and water. At the end of the experimental period, urine was collected without fecal contamination in ice-jacketed flasks at 4°C after 24 h using Sodium azide as a preservative for assessment of renal function. Cell debris and particulate matter were then removed from the urine samples by 1000 g centrifugation at 4°C for 30 mins. The supernatant was recovered. Aliquots of the supernatant were dialyzed and used for the assay of enzymes and protein. Estimation of stone forming constituents and other parameters were carried out with the ideal undialyzed sample.

After 24 h urine collection, the rats were sacrificed cervical decapitation. Prior to sacrifice, rats were anesthetized with Ketamine (22 mg/kg, i.m) and blood was collected by cardiac puncture into anticoagulant contained and anticoagulant free test tubes. Blood samples were kept at the room temperature for 30 min and then centrifuged at 3000 rpm for 10 mins. The supernatant was collected as serum and stored at −80°C for assay of enzymes and other biochemical assays. Plasma was collected by the centrifugation of the anti-coagulated blood. Kidneys were excised immediately and immersed in ice-cold physiological saline. The tissue was homogenized in ice-cold 0.01M Tris–HCl buffer, pH 7.4 to give a 10% homogenate and the aliquots of this homogenate were used for the assays. A section of renal tissue is kept aside for histopathological analysis.

Serum lipid status

Biochemical assays were performed using a Hitachi-912 chemistry analyzer from Roche. Cholesterol and triglycerides levels were analyzed using biochemical analysis kits from Roche.

High density lipoprotein (HDL)

Very low-density lipoprotein (VLDL) and LDL from serum were precipitated by phosphotungstate in the presence of magnesium ions. After removed by centrifugation, the clear supernatant containing HDL was used for the estimation of HDL cholesterol using cholesterol assay reagent kit from Roche.
**LDL calculation**

LDL cholesterol calculated according to the Friedewald formula\(^16\): \( \text{LDL} = \text{Total cholesterol} - (\text{Triglycerides}/5 + \text{HDL cholesterol}) \), and VLDL = Triglycerides/5

**Biochemical parameters in urine**

The urine volume and pH were determined. Calcium and magnesium\(^17\), phosphorus\(^18\), urea\(^19\), uric acid\(^20\) and protein (Bradford protein assay kit, Bio-Rad Laboratories, Hercules, CA, USA) concentrations were estimated using standard methods. Further, oxalate\(^21\) creatinine\(^22\), citrate\(^23\) were estimated. Urinary enzymes such as alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), acid phosphatase (ACP), \( \gamma \)-glutamyl transferase (\( \gamma \)-GT) were assayed by kit (Sigma, St. Louis, MO, USA) based methods.

**Isolation of brush-border membranes**

Rat renal brush-border fraction was prepared by the calcium precipitation method as described by\(^24\), with slight modifications\(^25\). The final pellet was rinsed three times to eliminate the calcium content thus avoiding activation of phospholipase, and resuspended in 100 mM mannitol/20 mM Hepes/Tris buffer (pH 7.4). The whole process was conducted at 4°C, and part of this membrane fraction was kept in liquid nitrogen for analyses not performed on the day of preparation.

**Assay of brush border membrane enzymes**

N-Acetyl glucosaminidase (NAG) and Leucine amino peptidase (LAP) activities were assessed by the method of\(^26,27\) with slight modifications, using 4-nitrophenyl-N-acetyl glucosaminide and L-leucyl -\( \beta \)-naphthylamide hydrochloride as the substrates and their activities were expressed as moles of \( p \)-nitrophenol formed/h/mg protein and \( p \)-nitroaniline formed/min/mg protein.

**Assays of transmembrane adenosine triphosphatases (ATPases) activity**

Sodium Potassium adenosine triphosphatases (\( \text{Na}^+, \text{K}^+ \) ATPase), Calcium adenosine triphosphatases (\( \text{Ca}^{2+} \) ATPase) and Magnesium adenosine triphosphatases (\( \text{Mg}^{2+} \) ATPase) were determined by the method of \([28-30]\), respectively. In all three cases, the enzyme activity is expressed as a function of inorganic phosphorus liberated, which is due to the breakdown of Adenosine triphosphate (ATP).

**Histopathological studies**

Histology of kidney was studied using hematoxylin and eosin (H and E). Portion of renal tissue was fixed in 10% buffered formalin. The washed tissues were dehydrated in the descending grades of isopropanol and finally cleared in xylene. The tissues were then embedded in molten paraffin wax. Sections were cut at 5 \( \mu \)m thickness, stained with H and E. The sections were then viewed under a light microscope (Nikon microscope ECLIPSE E400, Japan) for histopathological changes.

**Immunoblot analysis**

Isolated renal tissues (100 mg) were sliced very thinly and lysed by the addition of Radio immuno precipitation assay buffer (RIPA buffer) [150 mM NaCl, 50 mM Tris Hydrochloride (Tris–HCl) pH 7.4, 1 mM Ethylene diamine tetra acetic acid (EDTA), 1% NP-40 (IGEPAL) and 0.25% sodium deoxycholate] containing protease inhibitors: 1 mM Phenyl methane sulfonyl fluoride (PMSF), 1 \( \mu \)g/mL leupeptine and 1 \( \mu \)g/mL aprotinin; and phosphatase inhibitors: 1 mM Na\(_3\)VO\(_4\) and 1 mM NaF. Lysed tissue was briefly sonicated, incubated on ice for 1 h and centrifuged (13000 g for 45 min). Proteins were mixed with 6\( \times \) sample Laemmli buffer (0.35 M Tris–HCl, 4% Sodium dodecyl sulfate (SDS), 30% glycerol, 9.3% Di thio threitol (DTT), pH 6.8 and 0.01% Bromophenol blue) and warmed at 37°C for 15 min. Equal amounts of total protein (30 \( \mu \)g), determined using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with human serum albumin as a standard, were separated on a 7.5% SDS polyacrylamide gel and electro transferred to a Polyvinylidene fluoride (PVDF) membrane in Tris-glycine transfer buffer containing 20% methanol. Membranes were blocked in 3% non-fat dry milk in Phosphate buffered saline (PBS) for 1 h. Membranes were incubated overnight at 4°C with specific polyclonal antibodies THP (1:1000 dilution), albumin (1:500) and monoclonal anti rat IgG \( \kappa \) (1:2000) antibody. The membranes were subsequently washed and incubated with the anti-rabbit (1:5000), anti-mouse (1:2500) and anti- mouse (1:5000) secondary antibodies linked to horseradish peroxidase for 45 min, the band, was visualized using Enhanced Chemiluminescence (ECL) kit. Band intensity was measured by using Multianalyst (Bio-Rad).

**Data analysis**

The results are expressed as the mean \( \pm \) standard deviation (SD). Differences between groups were analysed by one-way analysis of variance (ANOVA) using the SPSS software package for Windows (Version: SPSS 20.0). Post hoc testing was performed.
for inter-group comparisons using the least significant difference (LSD) test; significance at $P$ values <0.05, 0.01 and 0.001 have been given in tables and figures.

**Results**

**Serum lipid analysis**

Table 1 shows the serum lipid profile in HCD fed experimental animals. The results reveal that there was a significant increase in all the lipid and lipoprotein parameters, with a concomitant decrease in HDL levels following HCD administration in young and aged rat groups when compared to their control groups. The alterations observed in aged control rats were found to be significantly increased when compared to that of young control rats.

**Analysis of urinary stone forming risk factors**

Table 2 shows the urinary stone forming risk factors in HCD fed experimental rats. The results of the study a significant increase in mean urinary volume together with a significant decrease in urine pH in aged HCD fed rats when compared with that of young control rats. Increased levels of urea, creatinine, uric acid, calcium, phosphorus, and oxalate were observed in the urine samples of aged HCD fed animals. We could find significant alteration in the concentration of magnesium, whereas we found a significant decrease in the levels of citrate and sodium in urine samples of aged HCD fed animals.

**Renal membrane integrity**

The assessment of cellular integrity was done by determining the activities of marker enzymes such as LDH, ALP, ACP and Gamma-glutamyl transpeptidase (GGT) in serum, as shown in (Table 3). We found a significant increase in the activities of these marker enzymes in the aged hypercholesterolemic group when compared to that of the young control group. Significant changes in the activities of these were observed in aged control rats when compared to young control rats.

**Determination of activities of brush border enzymes**

Table 4 showed the activities of brush border enzymes in renal tissues of young and aged HCD fed animals.
rats. The study revealed a significant decrease in the activities of NAG and LAP in aged HCD fed animals, whereas we could not find a significant decrease in the activities of these brush border enzymes in young HCD fed animals. A significant decrease in the activities of these enzymes was observed in the aged control group when compared to that of young counterparts.

Assessment of activities of membrane bound ATPases

Figure 1. shows the effect of HCD on activities of membrane bound ATPases in renal tissue of young and aged HCD fed rats. We observed significantly decreased activities of Na⁺K⁺ ATPase, Mg²⁺ and Ca²⁺ ATPases in renal tissue of young and aged HCD fed animals when compared to that of young control rats. The decrease observed in activities of these ATPases in aged control rats was found to be significant when compared with that of young control rats.

Histopathological examination of kidney/HCD induces renal damage

The fatty changes in the renal tissue histology of HCD fed aged and young rats are presented in (Fig. 2). Four to five sections were studied in each tissue. The results are found to be consistent in the sections studies in each tissue. The changes induced by high cholesterol diet are consistent in all six animals within the group. Hematoxylin-eosin stained kidney sections obtained from the HCD fed animals showed initial signs of renal damage characterized by mild tubular epithelial denudation more evident in aged HCD fed animals. No alterations were observed in the kidney of young control rats, whereas aged control rats showed mild fatty infiltrations.

Western blot analysis of THP, albumin and IgG κ in renal tissue

Figure 3 - 5 summarize the expression of renal THP, albumin and IgG κ, respectively, for each group of rats as determined by western blotting. The expression of renal albumin was significantly increased in renal tissue of aged and young HCD fed animals when compared to that of young control animals. The expression of THP was found to be significantly decreased in aged and young HCD fed rats when compared to that of young control rats. The expression of IgG κ was found to be significantly increased in aged HCD fed animals when compared to the young control group.

Table 3 — Activities of urinary marker enzymes in young and aged hypercholesterolemic stress induced rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
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<tbody>
<tr>
<td>LDH</td>
<td>478.2±39.8</td>
<td>672.2±61.1 ^i</td>
<td>693.7±69.4 ^i</td>
<td>802.5±89.2 ^i</td>
</tr>
<tr>
<td>ALP</td>
<td>86.7±9.6</td>
<td>93.0±9.3 NS</td>
<td>114.3±10.9 ^i</td>
<td>129.6±13.6 ^i</td>
</tr>
<tr>
<td>ACP</td>
<td>11.9±1.3</td>
<td>14.4±1.3 ^i</td>
<td>20.2±1.7 ^i</td>
<td>31.5±2.9 ^i</td>
</tr>
<tr>
<td>γGT</td>
<td>50.0±5.6</td>
<td>58.9±6.5 ^i</td>
<td>71.3±7.5 ^i</td>
<td>89.5±8.1 ^i</td>
</tr>
</tbody>
</table>

Units: ALP, ACP—µmoles of phenol liberated/ min/mg protein; LDH, Gamma GT—µmoles of pyruvate liberated/ min/mg protein. Group I—Young Control, Group II—Young HCD fed, Group III—Aged Control, Group IV—Aged HCD fed. Values are expressed as mean ± SD for six rats in each group. Values are compared using Group I as control. ^P < 0.001, * P < 0.05, NS: Non-Significance

Table 4 — Determination of activities of brush border membrane enzymes in the renal tissue of young and aged HCD fed rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAG</td>
<td>15.4±1.7</td>
<td>14.4±1.6 ^NS</td>
<td>12.1±1.3 ^i</td>
<td>7.6±0.8 ^i</td>
</tr>
<tr>
<td>LAP</td>
<td>1.0±0.1</td>
<td>0.9±0.1 ^NS</td>
<td>0.9±0.1 ^i</td>
<td>0.7±0.1 ^i</td>
</tr>
</tbody>
</table>

Units: NAG—µmoles of N-acetyl-β-D-glucosaminide liberated/ min/mg protein; LAP—µmoles of p-nitroaniline liberated/ min/mg protein. Group I—Young Control, Group II—Young HCD fed, Group III—Aged Control, Group IV—Aged HCD fed. Values are expressed as mean ± SD for six rats in each group. The values are compared using Group I as control. ^P < 0.001, * P < 0.05, NS: Non-Significance
to that of young control rats, but we could not observe any significant alterations in young HCD fed animals when compared to that of its younger counterparts.

Discussion

The disruption of lipid homeostasis is a well-established phenomenon of the complex metabolic changes that occur with normal aging. The relationship between lipid and fatty acid related hyperoxaluria and hypercalciuria have been documented in earlier studies. There are reports suggesting the prevalence and recurrence of lithiasis...
in aged individuals. However, little direct evidence exists to propose a possible mechanism by which aging itself accounts for stone formation. It is also necessary to decipher whether hypercholesterolemia augments the propensity of aged people to form urinary stones, as metabolic derangements and urinary electrolyte imbalance may stem from altered lipid metabolism and cardiovascular disease (CVD). This prompted us to evaluate the plausible implications of hypercholesterolemic stress in exacerbating renal stone forming risk factors in aged male wistar albino rats.

Concerning serum lipid profile in young and aged rats stressed with hypercholesterolemia, a significant increase in total cholesterol, LDL, triglycerides and VLDL levels were observed when compared with young and aged rats fed with a normal diet, while a significant decrease in HDL level was recorded. These results were in accordance with the results of who added that dietary cholesterol and cholic acid supplementation increase the synthesis of fatty acids and triacylglycerol in rat liver and enhance cholesterol absorption. High cholesterol diet feeding was also found to increase total cholesterol and LDL level significantly. The results of the present work revealed that elevated lipid levels could be due to the collapse of oxidative defense systems and augmented inflammation in the hypercholesterolemic conditions.

Urinary chemistry analysis with respect to the stone-forming minerals will provide a good indication of the risk of stone formation. The results of the present work showed a significant rise in mean urine volume and decline in urinary pH of aged HCD fed animals when compared to young control rats. An age related decline in glomerular filtration rate (GFR), an increase in relative volume of the neural lobe, a slight decrease in the amount of neurosecretory material in the neural lobe and an increase in plasma anti-diuretic hormone (ADH) concentration could be the possible reasons for eventual developmental of polyuria in those animals. Indeed, our results are in unison with the previous reports depicting that obese individual with nephrolithiasis are associated with lowered urinary pH. The levels of urea, creatinine and uric acid were increased considerably in the urine samples of aged excess cholesterol diet fed rats which indicate glomerulopathy due to severe hyperlipidemia.

Gradual increases in stone forming minerals like urinary calcium, phosphorus and oxalate have been observed in aged hypercholesterolemic rats. Elevated urinary calcium excretion during hyperlipidemia is a repercuision of diminished calcium entry into bones, rather than calcium efflux from bones. Increased phosphorus excretion along with oxalate stress seems to provide an environment appropriate for stone formation by forming calcium phosphate crystals, which epitaxially induces calcium oxalate deposition. Moreover, obesity related hyperuricemia also promotes endothelial dysfunction, oxidative metabolism, platelet adheresiveness, hemorheology and aggregation, further exaggerating renal tubular injury and nephrolithiasis in aging.

Several inhibitors of crystallization such as citrate, magnesium and others are found in normal urine. Magnesium, diminishes supersaturation of calcium oxalate as well as the growth and the nucleation rates of calcium oxalate crystals in hyperoxaluric rats. In this study, we could not observe any significant alteration in the concentration of magnesium excreted in the urine of young and aged HCD fed rats. Interestingly, decreased levels of citrate in aged hypercholesterolemic rats demonstrated an increased risk for stone formation, as hypocitraturia in elderly is coupled with proximal tubular cell acidification. Such tubular acidification increases apical membrane citrate uptake as well as cellular citrate metabolism.

Proteinuria is an important manifestation of renal proximal tubular dysfunction. Increased protein excretion during aging as well as hyperlipidemia has already been reported in earlier studies. Our study also showed a marked elevation in renal albumin expression in young and aged hypercholesterolemic rats in comparison to young control rats. This could be due to high fat intake stimulated renal tubular accretion of albumin and oxidative damage generated by free radicals leading to increased permeability of albumin resulting in proteinuria has reported that the renal activation of albumin gene results in acute kidney injury. But, aged control rats did not reveal a significant increase in albumin expression when compared to young control rats which are contrary to studies done by. The reason for such altered albumin expression is unknown.

Most of the urinary enzymes originating in the renal tissue are localized to distinct regions, and cellular components of the nephron, thereby research pertaining to these enzymes will reveal the pathological status of the kidney. Enzymes such as LDH and ALP leak out from the damaged tissues to the blood stream, when the cell membrane becomes permeable or rupture. Interestingly, the activities of LDH, ALP,
ACP and γ-GT in urine were increased to a considerable extent in young and aged HCD fed rats compared to young control rats suggesting that hypercholesterolemia during aging might have exerted oxidative stress from the oxidation of major biomolecules and diminished antioxidant defense system. The enhanced activities of these enzymes in hypercholesterolemia were concurrent with the studies of 36. The enhanced urinary excretion of these enzymes in hypercholesterolemia were concurrent antioxidant defense system. The enhanced activities of the oxidation of major biomolecules and diminished during aging might have exerted oxidative stress from young control rats suggesting that hypercholesterolemia extent in young and aged HCD fed rats compared to the reason for the decreased renal activity of Na⁺-K⁺ ATPase and Protein kinase C (PKC) activity 57 could also be and an age-related increase in renal oxidative stress 56. We observed a significant decrease in LAP and NAG activities in aged animals fed with cholesterol diet. At the same time, we could not perceive considerable alteration in activities of these enzymes in young cholesterol diet fed rats which imply an age dependent modulation in renal brush border membrane enzymes.

Our data reinforce the concept that cholesterol enrichment increases organic cation transport in renal brush-border membranes 57. We observed a significant decrease in the activities of the renal Na⁺-K⁺ ATPases in hypercholesterolemic diet fed young and aged rats when compared to young control animals. This may be due to lipid peroxidation occurring after cholesterol accretion of the cell membrane. Higher basal serine-phosphorylation of the α1-subunit of Na⁺-K⁺ ATPase in the proximal tubules of aged rats and an age-related increase in renal oxidative stress and Protein kinase C (PKC) activity 57 could also be the reason for the decreased renal activity of Na⁺-K⁺ ATPase. Moreover, Ca²⁺ ATPase and Mg²⁺ ATPase activities were also reduced in aged hypercholesterolemic rats which could be attributed to the oxidation of membrane lipids and proteins. The inability of aged kidneys to maintain acid-base balance when subjected to acid load (as seen in hyperlipidemia) could be the possible reason for the decreased availability of ATPases in the basolateral membrane of the tubular cells 58. High protein and lipid intake impair H⁺ secretion imposing acid load to the renal tubular cells exacerbating the renal damage.

THP is the most abundant protein in the human urine and is synthesized and secreted by epithelial cells of the thick ascending limb of the loop of Henle and early distal convoluted tubule 59. The kinetic properties of THP are governed by factors such as citrate, calcium, oxalate, uric acid, and oxidative damage. In normal conditions, citrate not only maintains the alkalinity of the urine and complexes with calcium, thereby prevents it from reacting with oxalate, it also maintains THP in its monomeric form, which is essential for its inhibitory activity. During urolithic condition, due to reduced excretion of THP, it tends to aggregate and act as a heterogenous nucleator of calcium oxalate 60. Numerous clinical and experimental studies have indicated the involvement of THP in several forms of inflammatory kidney disease 61. Reports are stating that urinary disaggregated THP concentration is decreased in the elderly, and that aggregate and THP is increased compared to younger adults. The aggregated THP concentration is decreased in the elderly during episodes of urinary tract infection 62. Hypercholesterolemia and other cardiovascular risk factors resulted in decreased THP levels and proteinuria in nephritic syndrome associated children 63. We also found decreased protein expression of THP-1 in aged HCD fed rats and the probable mechanism for such a decrease could be a mild tubular and nephrotic damage on ingestion of high cholesterol diet.

Renal inflammation and microalbuminuria can be indicated by the increased renal expression and urinary excretion of certain plasma proteins such as IgG, transferrin and ceruloplasmin, etc. 64. The current investigation revealed a significant elevation in the protein expression of IgG κ light chain in aged and aged hypercholesterolemia induced rats. But we did not observe any appreciable variation in young HCD rats which imply that aging coalesced with hypercholesterolemia intensify tubular injury. Thus these data suggest that short term hypercholesterolemic stress to aged rats may alter the expression of extra cellular matrix (ECM) proteins like THP, albumin and the expression of renowned tubular injury marker, IgG κ light chain protein.

The renal abnormalities induced by senescence associated hypercholesterolemia are further confirmed by histopathological findings. Mild tubular epithelial denudation with casts and marked fatty infiltrations and a minimal level of necrosis were also seen in the renal tissue of aged HCD-fed rats, which can be attributed to lipemic-oxidative injury. Besides, young
HCD fed animals also showed marked fatty changes when compared to young control rats. These results demonstrate that diet-induced hypercholesterolemia is associated with increased microvascular density in the renal cortex, which precedes signs of overt renal morphological damage. These alterations may potentially affect regulation and spatial distribution of intra renal blood flow in hypercholesterolemia which may get aggravated during aging condition and may participate in renal disease progression. Previous studies have demonstrated that hypercholesterolemia predisposes renal tubular cells to hypoxic injury, wherein hypercholesterolemia mediates a direct effect on epithelial tubular cells, which thus become more susceptible to ischemic injury.4

Conclusion
In conclusion, age associated hypercholesterolemia has a strong association and can act as an independent risk factor for kidney stone formation. The present study demonstrates that combinatorial stress of aging and hypercholesterolemia accentuate their individual pathophysiological implications on renal function and may precede the development of urolithiasis, which is obvious from the excretion pattern of urinary enzymes and intensified the process of crystal deposition, thereby formation of stones. Our analysis underscores the advancement of renal injury when kidneys are exposed to both risk factors concurrently. Furthermore, aging process also heaps on the vulnerability of structural and functional modifications of the macromolecules like THP, disrupting their normal inhibitory activities and these alterations become more severe in the presence of hyperlipidemia.

Although it is difficult to draw inference for humans from animal studies, our investigation has important implications for clinical care and public health as Age-related lipid anomalies are so common. So, a better understanding of pathophysiological mechanisms underlying the inception and progression of kidney stones in senescence related hypercholesterolemia may enable the development of better and earlier therapeutics, in the near future. This study also emphasizes the importance of lifestyle modification to prevent the secondary health complications during aging.

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