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EXPERIMENTAL STUDY

High cholesterol diet leads to oxidant load and peroxidation in the rabbit kidney tissue

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ABSTRACT
OBJECTIVES: The aim of this study was to investigate possible effects of high cholesterol diet on oxidant/antioxidant status in rabbit kidney tissues.
BACKGROUND: Although a number of experimental animal models have suggested that hyperlipidemia is associated with progressive kidney failure data remain sparse on the role of dietary cholesterol intake on kidney disease.
METHODS: Twelve male New Zealand albino rabbits were randomly divided into two groups (control and cholesterol). Both groups were fed on a standard laboratory diet. Animals in the cholesterol group additionally received cholesterol (1 g/kg/day), orally. The study period was 12 weeks. Activities of catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), nitric oxide synthase (NOS), xanthine oxidase (XO), paraoxonase (PON), adenosine deaminase (ADA) enzymes and levels of malondialdehyde (MDA) and nitric oxide (NO) were measured in kidney tissue samples. Histological examination of the kidney tissue samples was also done.
RESULTS: SOD, GSH-Px and XO enzyme activities were found to be decreased and NOS and PON activities increased significantly in cholesterol group compared to controls. As an indication of oxidation, MDA levels were found to be increased in cholesterol group. Histological examination revealed some derangements in the kidney tissue.
CONCLUSION: High cholesterol diet creates oxidant load and causes peroxidation, which in turn, leads derangements in the rabbit kidney tissue *(Tab. 2, Fig. 2, Ref. 69)*. Text in PDF *www.elis.sk.*
KEY WORDS: cholesterol, oxidant / antioxidant system, kidney, rabbit.

Introduction

Lipid toxicity hypothesis has been supposed for the kidney diseases for some time (1), which argues that lipid abnormalities can contribute to glomerulosclerosis. Cholesterol supplementation to the diets has been found to lead to focal and segmental glomerulosclerosis (2–4), and lipid deposits are found in focal segmental sclerosis in human renal biopsies (5). It has been argued that hyperlipidemia can be both consequence and cause of the progression of chronic kidney disease (CKD). This process is accepted to be analogous to coronary atherosclerosis (6, 7). Therefore, statins have been used to prevent CKD for some time. The effect of statins remains controversial although many clinical studies indicate that correcting dyslipidemia is associated with slow progression of renal failure (8–10). It has been postulated that lipids activate various growth factors causing mesangial cell proliferation (11, 12) and inflammation in glomerulonephritis (13). In fact, several hemodynamic and metabolic factors have been shown to contribute to the progression of renal diseases (14). Hypercholesterolemia was found to induce or exacerbate glomerular injury in mammals. Data suggest that lipids and lipoproteins affect not only glomeruli but also the tubulointerstitium (15).

Oxidative stress can be considered an imbalance in the reactive oxygen species (ROS) production/degradation rate ratio. Under normal conditions, ROS are produced in mammalian cells during energy production in mitochondria by reducing oxygen during aerobic respiration (16). Excessive ROS levels can produce cellular damage by interacting with biomolecules and thus have negative effects on tissue function and structure. As such, they are implicated in different pathological situations (17) including a variety of renal diseases (18). Renal dysfunction is found to be frequently associated with oxidant stress (19–21). It has been established that ROS increase in a graded manner as renal function deteriorates, even inverse correlations between different markers of oxidative stress and glomerular filtration rate have been reported (19, 22).

Oxidants and oxidative modifications have been suggested to play a major role in permanent tissue damage (23). The detrimental effects of ROS have also been documented in renal parenchyma, mesangial cells in culture, and on matrix components (24–26). In hyperlipoproteinemia, cell membranes and the extracellular matrix can change their lipid composition and be more prone to radical generation (3). Hypercholesterolemia has been reported to increase superoxide anion production in endothelial cells (27). Oxidative

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modification of lipids is able to lead to a self-perpetuating cycle of oxygen radical generation and modification of proteins (28).

In the body, there are oxidant-producing and consuming metabolic pathways and systems. One of the oxidant producing ways is the purine catabolic pathway, the last product of which is uric acid by a reaction catalyzed through xanthine/xanthine oxidase (XO) system. Antioxidant systems however consist of enzymatic and non enzymatic mechanisms. Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) enzymes are the main components of this system. Nitric oxide synthase (NOS) and paraoxonase (PON) enzymes are also accepted as auxiliary antioxidant enzymes. Glutathione, uric acid, ascorbic acid, bilirubin etc. are the non enzymatic antioxidants.

Of these, adenosine deaminase (ADA) is an enzyme (EC 3.5.4.4) involved in purine metabolism. It is needed for the breakdown of adenosine and for the turnover of nucleic acids in tissues (29). ADA irreversibly deaminates adenosine, converting it to the related nucleoside inosine by the substitution of the amino group for a hydroxyl group. Inosine can then be deribosylated to hypoxanthine by another enzyme called purine nucleoside phosphorylase (PNP). Hypoxanthine is first converted to xanthine and then to uric acid by xanthine oxidase (XO) enzyme. In the last reaction, superoxide radical is produced as a byproduct, which creates oxidant load to the living cells if it is not dismutated by SOD enzyme.

XO (EC 1.17.3.2) is an enzyme which produces reactive oxygen species as described above. It plays an important role in the catabolism of purines in some species. This enzyme catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid, the end product of purine catabolic pathway (30).

SODs (EC 1.15.1.1) are enzymes that alternately catalyze the dismutation of the toxic superoxide ($O_2^{\bullet-}$) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and causes cell damage. Hydrogen peroxide is also damaging, but less so, and is degraded by other enzymes such as CAT. Thus, SOD is an important antioxidant defense in nearly all living cells exposed to oxygen (31).

GSH-Px (EC 1.11.1.9) is an enzyme catalysing conversion reaction of H_2O_2 to O_2 and water. It plays significant physiological role to protect the organism from oxidative damage. The main reaction that GSH-Px catalyzes is: $2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$ where GSH represents reduced monomeric glutathione and GS–SG represents glutathione disulfide. The biochemical function of GSH-Px is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water (32).

CAT (EC 1.11.1.6) is found in nearly all living organisms exposed to oxygen. It also catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cells from oxidative damage by ROS (33). SOD, GSH-Px and CAT all together form main enzymatic antioxidant defense mechanism in the living cells.

PON (EC 3.1.8.1) was identified as an antioxidant enzyme using organophosphates as substrate. There are three known genotypic forms of paraoxonases; PON1, PON2 and PON3. The differences between them lie in their location and activity. They all protect cells against oxidative damage. They are implicated in lowering the risk of developing atherosclerosis as they function to prevent the formation of atherogenic oxidised-LDL, the form of LDL present in foam cells of an atheromatous plaque (34, 35).

NOSs (EC 1.14.13.39) are a family of enzymes catalyzing the production of nitric oxide (NO) from L-arginine. Of these isoenzymes, iNOS and nNOS are soluble and found predominantly in the cytosol, while eNOS is membrane associated. In mammals, the endothelial isoform is involved in regulation of cardiac function and angiogenesis. NO produced by eNOS has been shown to be a vasodilator. The neuronal isoform is involved in the development of nervous system. iNOS is involved in immune response (36).

Malondialdehyde (MDA) is the end product of lipid peroxidation with the formula $CH_2(CHO)_2$. The structure of this species is more complex than this formula suggests. This reactive species occurs naturally and is a marker for oxidative stress. The degree of lipid peroxidation can be estimated by the amount of malondialdehyde in tissues (37).

Although a number of experimental animal models have suggested that hyperlipidemia is associated with progressive kidney failure (1, 38, 39), data remain sparse on the role of dietary cholesterol intake on kidney disease. Therefore, this subject needs further studies including various oxidant/antioxidant system parameters in the kidney tissues from animals fed on high cholesterol diet.

Materials and methods

This study was approved by local Animal Experiments Ethics Committee (G.Ü.ET-12.091). Twelve male New Zealand albino rabbits (provided by Experimental Animals Laboratory of Public Health Establishment) were randomly divided into two groups (control and cholesterol). Both groups were fed on a standard laboratory diet. Animals in the cholesterol group additionally received cholesterol (1 g/kg/day), orally. Study period was 12 weeks. One of the animals in the cholesterol group died during the experimental period so that the study was terminated with 11 animals. Animals were kept in standard polycarbonate cages with stable temperature (21–24 °C) and humidity (30–40 %) throughout the study period.

Blood samples were obtained at the beginning and at the end of the study period of 12 weeks. At the end of the study period after blood samples collection, animals were sacrificed under anesthesia (Xylazine 5 mg/kg i.m, Ketamine 45 mg/kg i.m). After sacrification, kidneys were removed and kept in –80 °C until analyses. Parts of the tissues were put into formaline solution (10 % w/v) for histological investigation.

Blood samples were sent to routine biochemistry laboratory for BUN, creatinine, total cholesterol and triglyceride levels. For the tissue analyses, kidney tissues were first washed with water and then homogenized in physiological saline solution (20 % w/v). After centrifugation at 5000 rpm for 30 min, upper clear supernatants were removed to be used in the analyses (40).

Protein amount was measured by Lowry method (41). Tissue cholesterol, triglyceride and uric acid levels were analyzed by using routine laboratory methods. ADA (42), XO (43), SOD (44), GSH-

Px (45), CAT (46), PON (47) and NOS (48) enzyme activities were measured as described. NO levels were measured by nitroprussid method (49) and MDA levels by thiobarbituric acid method (50).

Results were expressed as mg/dl for blood analysis parameters, as specific activity (unit/mg protein) for enzymatic analyses, and as mg or mmol/g tissue weight for protein, uric acid, cholesterol, triglyceride, MDA and NO levels in kidney tissue.

For light microscopic histological examination, the samples were obtained from the kidney, fixed in 10 % neutral buffered formaline solution for 3 days. Tissues were washed in flowing water and were dehydrated with rising concentrations of ethanol (50 %, 75 %, 96 % and 100 %). After dehydration, specimens were put into xylene to obtain transparency and were then infiltrated with and embedded in paraffin. Embedded tissues were cut into 5-µm thick sections by Leica RM 2125 RT (Bensheim, Germany). Sections were stained with hematoxylin–eosin (H–E) and Mallory–Azan (MA) dyes. Samples were examined under a light microscope (Zeiss Axio Scope A1 Oberkochen, Germany).

In the statistical evaluation, SPSS 15 for Windows package program was used (Student's t- test) and values lower than $p < 0.05$ were evaluated as significant.

Tab. 2. Parameters measured in the kidney tissues from control and cholesterol groups.

PARAMETERS	Control Group (n=6)	Cholesterol Group (n=5)	P values
Total Cholesterol (mg/g tissue)	5.44±0.62	8.52±0.94	<0.05
Triglyceride (mg/g tissue)	15.96±2.20	15.62±2.06	ns
Total Protein (mg/g tissue)	13.8±2.12	14.48±3.02	ns
Uric acid (mg/g tissue)	0.100±0.012	0.139±0.014	<0.01
NO (mmol/g tissue)	48.54±5.20	51.75±6.72	ns
PON (IU/mg protein)	3.05±0.34	4.32±0.50	<0.05
NOS (IU/mg protein)	0.871±0.110	0.978±0.120	<0.05
SOD (U/mg protein)	919.1±110.2	792.8±85.3	<0.05
GSH-Px (mIU/mg protein)	35.67±4.5	19.86±2.4	<0.01
CAT (IU/mg protein)	119.25± 14.50	101.10±12.52	ns
ADA (IU/mg protein)	6.03± 0.78	6.99±0.84	ns
XO (mIU/mg protein)	61.7±7.1	54.5±6.2	<0.05
MDA (nmol/g tissue)	15.05±1.82	31.39±4.21	<0.01

ns – non significant ($p > 0.05$)

Results

Results are given in the Tables 1 and 2. As seen from Table 1, significant differences were observed for some parameters in the cholesterol group. In particular, blood cholesterol and triglyceride levels were significantly higher in post- (at the end of the study period) samples as compared with pre- (at the beginning of the study period) samples.

As to the kidney tissue parameters; total cholesterol, uric acid and -as an indication of oxidation- MDA levels were found to be significantly higher in the cholesterol group compared to those of the controls (Tab. 2). SOD, GSH-Px and XO enzyme activities were found to be decreased and NOS and PON activities increased significantly in cholesterol group compared to controls (Tab. 2). However, there were no significant differences between the groups regarding ADA and CAT enzyme activities and NO levels.

In the histological examination of cholesterol group kidney tissue samples: enlargement of urinary space (Fig. 1b), dilatation of peritubular venules and distal convoluted tubules (Fig. 1b, c), enhanced fibrosis especially around the proximal convoluted tubules (Fig. 2 b, c) in cortex; narrowed urinary space of glomerulus especially in outer cortex (Fig. 1c); and hypertrophy of columnar cells of collecting duct, capillary dilatation, necrotic area (Fig. 1e, f) and adipose tissue (Fig. 2a) in interstitium were observed.

Discussion

Several hemodynamic and metabolic factors have been shown to contribute to the progression of renal disease in animal models and in humans (14). Hyperlipidemia, specifically hypercholesterolemia, can induce or exacerbate glomerular injury in mammals (51, 52). Data from animal experiments and clinical studies suggest that lipids and lipoproteins affect not only glomeruli but also the tubulointerstitium (15). Interstitial fibrosis and tubular atrophy have been documented in both female and male hypercholesterolemic rats without primary glomerular disease (15). In cell culture experiments, oxygen radicals were identified as messengers in this regard (53).

Rats fed on a high-saturated animal fat diet were established to have decreased glomerular number and size when compared with rats fed on a high fat diet of canola oil, which is high in monounsaturated fat (54). In rats fed on different oils, or butter, decline in glomerular filtration rate (GFR) was found to be greatest in those receiving butter, perhaps because of a higher percentage of saturated fat. It has been convincingly demonstrated that rats with

Tab. 1. Blood urea nitrogen (BUN), creatinine (CR), triglyceride (TG) and cholesterol (CH) levels (mg/dl) of the control and cholesterol groups in pre and post blood samples.

Groups	Pre-samples Median (min–max)				Post-samples Median (min–max)				Statistical evaluation (*P* values) Pre vs post			
	BUN	CR	TG	CH	BUN	CR	TG	CH	BUN	CR	TG	CH
1 *(n=6)*	10.00 (7–18)	0.98±0.14	67.50±16.29	47.00 (21–67)	18.50 (14–23)	0.97±0.03	93.33±16.15	181.50 (124–233)	n.s.	n.s	n.s	0.028
2 *(n=5)*	14.00 (9–22)	1.01±0.13	83.20±32.213	39.00 (17–48)	15.00 (10–19)	0.87±0.27	623.0±85.2	1277.00 (1213–1830)	0.043	n.s	<0.01	0.043
Statistical evaluation (*P* values)												
1 vs 2	n.s	n.s	n.s	n.s	n.s	n.s	<0.05	<0.004				

1 – Control group; 2 – Cholesterol group, Pre-samples – Blood samples collected at the beginning of the study period, Post-samples – Blood samples collected at the end of the study period, n.s – non significant ($p > 0.05$)

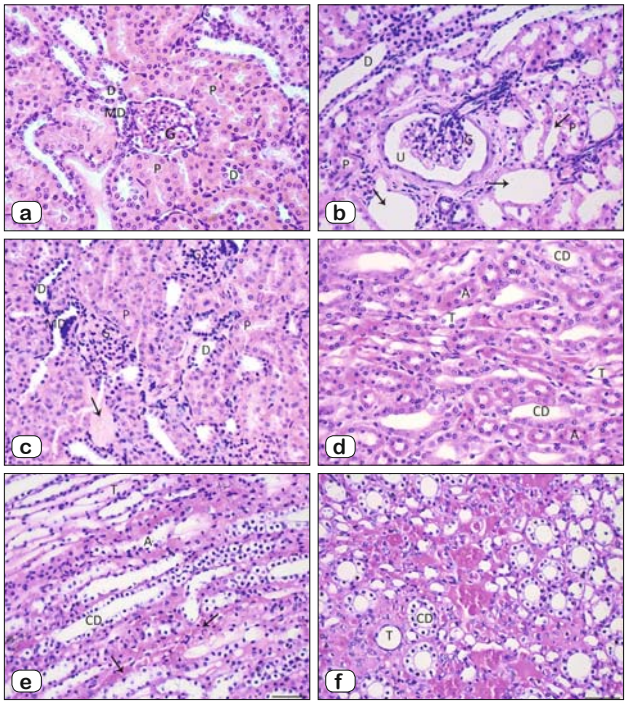

Fig. 1. Micrographs taken from the histological preparations of kidney tissues. Hematoxylin–Eosin (HE). a) Major histological features of control group. G – glomerulus, MD – macula densa, P – proximal convoluted tubules, D – distal convluted tubules. HE x 400. b) Urinary space (U) is enlarged and distal convoluted tubules are dilated in cholesterol group. HE x 400. c) Urinary space of glomerulus is observed as narrow and peritubular venules (arrow) dilated in some slides of cholesterol group. HE x 400. d) Medulla of control group is seen in normal appearance. A – thick ascending limbs, T – thin descending and ascending limbs of loops of Henle, CD – collecting ducts. HE x 400. e) Pale columnar cells of collecting ducts are hypertrophic and capillaries are dilated in medulla of cholesterol group. HE x 400. f) Necrotic area is observed in interstitium of the medulla of the cholesterol group. HE x 400.

chronic hyperlipidemia and hypercholesterolemia develop glomerulosclerosis in kidneys (51, 55, 53). Hyperlipidemia was also supposed to be associated with chronic tubulointerstitial damage (15, 56). Several lines of evidence indicate that oxidant stress is a pathogenic factor in lipid-induced nephropathy. By immunohistology, oxidatively modified lipoproteins were demonstrated in focal segmental glomerulosclerosis in rat and humans (57–59). However, the steps leading to oxidative modification of proteins and lipoproteins have not been elucidated in detail yet.

Oxidative stress can be considered as an imbalance in the reactive oxygen species (ROS) production/degradation ratio. Under normal conditions, ROS are produced in mammalian cells during energy production in mitochondria (16). In addition, a variety of enzymatic and non-enzymatic sources of ROS exist in vascular vessels as well as different tissues (60). An uncoupling of nitric oxide synthase can also contribute to ROS production (61). ROS levels are maintained within a normal range by scavenging through various enzyme activities, and other components (23). In fact, ROS are part of the organism's unspecified defense system. However, excessive ROS levels can produce cellular damage by interacting with biomolecules, and thus have negative effects on tissue function and structure. As such, they are implicated in different pathological situations including a variety of renal diseases (18). In a study, it has been demonstrated that oxygen radical generation is increased in the glomeruli of hypercholesterolemic, uninephrectomized male Wistar rats. This increase was evident as soon as the incidence of glomerulosclerosis increased. In a study, in contrast to the dominant role of NADH/NADPH oxidase activity, no change in the oxidant enzyme systems was established in the glomerular membranes of rats with Heymann nephritis (62). However, xanthine oxidase infused into rabbit kidneys was found to initiate glomerular damage (63). In hypercholesterolemic rabbits, a two-fold elevation of plasma xanthine oxidase activity has been reported (64). It was also shown that xanthine oxidase caused enhanced superoxide production of aortic vessel rings. It has been suggested that increased glomerular oxygen radical production was mainly caused by xanthine oxidoreductase (65). The increased ROS generation in glomeruli of hyperlipidemic rats without glomerulonephritis seems to result primarily from elevated xanthine oxidase activity. Data on any direct effect of hypercholesterolemia on rat kidneys with glomerular immune complex disease have not yet been reported. Hyperlipidemia significantly aggravated tubulointerstitial damage in uninephrectomized rats with glomerulonephritis. A disturbed balance between oxidant

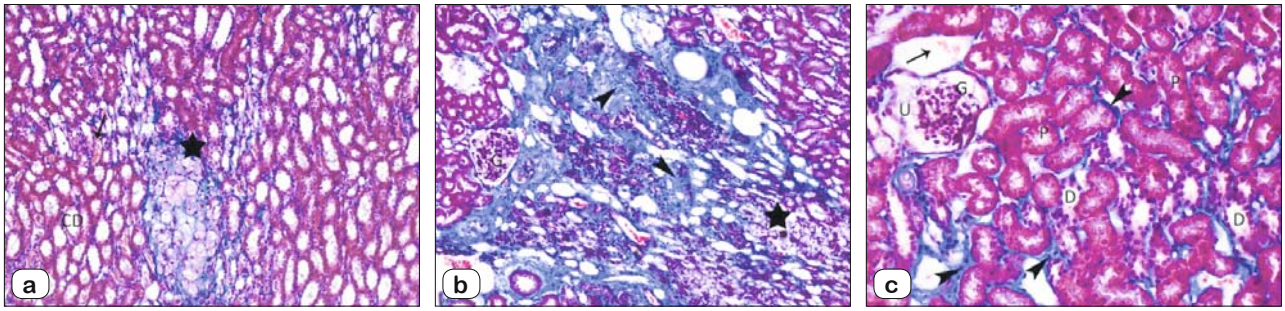

Fig. 2. Micrographs taken from the histological preparations of cholesterol group kidney tissues. Mallory–Azan (MA). a) Adipose tissue in medulla. MA x 200. b) Enhanced fibrosis in cortex. MA x 200. c) Peritubular fibrosis. MA x 200. G – glomerulus, U – urinary space, P – proximal convuluted tubule, D – distal convuluted tubule, arrow – peritubular capillaries and draining venules, arrow head – peritubular fibrosis, star – adipocytes, CD – collecting ducts.

and antioxidant enzyme activities may have been involved. The generation of ROS and the activity of xanthine oxidase in the cortical tubulointerstitium rose significantly in hyperlipidemia. This lack of synchrony between chronic irreversible damage and oxidant biochemical parameters may not contradict a pathogenetic interaction. It may be surmised that an altered oxidant/antioxidant balance probably precedes severe damage and that a more or less constant level of oxidant stress can maintain ongoing damage. In addition, antioxidant enzyme activity in the cortical tubulointerstitium tended to decrease in nephritic rats. Renal dysfunction is frequently associated with oxidative stress (19–21). It appears that ROS increase in a graded manner as renal function deteriorates, as different studies have reported inverse correlations between different markers of oxidative stress and glomerular filtration rate (22, 66). It was postulated that lipid-induced tubulointerstitial disease might occur via oxygen radical generation. As the ratio of oxidant and antioxidant enzyme activities determines the oxidant status of renal cells and tissues (67), a failure of a compensatory response in antioxidant enzyme activities or an increase in oxidant enzyme activity are both suggested to contribute to renal oxidant stress.

Nevertheless, it has to be stressed that the present data do not provide unfailing evidence for a causal relation between lipid-induced oxygen radical generation and chronic tubulointerstitial injury. It has been postulated that oxidatively modified proteins may contribute to the progression of irreversible renal damage (68). In hyperlipidemic rats, proteinuria was significantly higher than in rats with normolipidemia. In a recent study, it has been found that higher saturated fat intake is significantly associated with the presence of high albuminuria (69).

In summary, it has been suggested that oxidant stress significantly contributed to these chronic degenerative processes by enhanced glomerular generation of ROS. In mesangioproliferative glomerulonephritis, hyperlipidemia was found to induce chronic tubulointerstitial damage through a shift of oxidant/antioxidant enzyme activity ratio. It has been found that in this situation, xanthine oxidoreductase activity was raised, and antioxidant enzyme activities decreased. In parallel, urinary excretion of oxidatively modified proteins was elevated. The ability of hyperlipidemia to modulate renal disease and damage different renal compartments seems to be dependent on the primary renal disease and increased oxidant stress. Therefore, statins in preventing progression of CKD have been used to prevent CKD for some time. However, the effect of statins remains controversial although many clinical studies indicate that correcting dyslipidemia associated with kidney disease may slow progression to renal failure (8–10). The present findings demonstrate that hyperlipidemia increased oxidant stress. A rise in the ratio of oxidant to antioxidant enzyme activities seems to be of importance in this regard. However, there is still no accordance among the results in this regard. Therefore, a comprehensive study aiming to establish the status of ROS producing and consuming systems and their internal metabolites is needed. The present study was conducted for this aim.

Looking at our results, it seems that total cholesterol level is significantly increased in the blood and kidney tissues from rabbits fed on cholesterol-rich diet. However, no differences existed in triglyceride and protein levels in the kidney tissue. There were also no differences between blood urea nitrogen and blood creatinine levels of the groups, showing no impairment in renal function. Kidney tissue uric acid level was higher in the cholesterol group relative to controls, which indicated accelerated purine catabolism. There was no difference between tissues NO levels of the groups whereas NOS activity seemed increased in the cholesterol group. Similarly, PON activity also increased in the cholesterol group relative to controls. The increases in the NOS and PON activities might arise from an attempt to compensate changed NO level and weakened antioxidant system because SOD, and in particular GSH-Px activities significantly lowered in the cholesterol group. As to the MDA level, it seems that there is an oxidant stress and accelerated oxidation reactions in the cholesterol group. It is possible that the main reason of increased MDA level is weakened antioxidant system instead of oxidant load as purine nucleotide enzymes seem not to increase in the cholesterol group. At least, our results indicated no increase in superoxide radical production due to lowered XO activity. However, we cannot discard another hypothesis implicating increased uric acid as an effective inhibitor for XO enzyme. It is also possible that factors leading to increased uric acid production like increased purine breakdown may cause increased superoxide production in the kidney tissue from rabbit fed on high cholesterol diet, which can also lead to oxidant stress and accelerated peroxidation reactions.

To sum up, our results suggest that high cholesterol feeding leads to oxidant load and peroxidation in the kidney tissue possibly through weakened antioxidant system. We suggest that antioxidant support may be helpful for the kidney health of the subjects fed on high cholesterol diet.

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