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Hyperlipidemia Enhanced Oxidative Stress and Inflammatory Response Evoked by Renal Ischemia/Reperfusion Injury

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Abstract: This study was designed to investigate possible effect of hyperlipidemia on renal ischemia/reperfusion injury in rat. Male Wistar albino rats were divided into 4 groups. Hyperlipidemia was induced by cholesterol (500 mg kg^{-1} p.o.) feeding in hydrogenated ground nut oil (as a vehicle) for 4 weeks. At the end of 4th week renal ischemia/reperfusion injury was perform by occlusion of both renal vascular pedicles for 60 min, followed by 24 h reperfusion. During reperfusion period, blood and urine were collected for biochemical analysis. Both kidneys were isolated, one kidney for histopathological evaluation and one for tissue parameters. The lipid peroxidation, xanthine oxidase activity and nitric oxide level in renal tissue were significantly increased after I/R in hyperlipidemic rats compared to ischemia/reperfusion in normal rats. Antioxidant enzymes like reduced glutathione, superoxide dismutase and catalase were significantly reduced after ischemia/reperfusion in hyperlipidemic rats compared to normal rats. Serums TNF-α level and myeloperoxidase activity in renal tissue, were also significantly increased after ischemia/reperfusion in hyperlipidemic rats. Furthermore, hyperlipidemic rats that underwent ischemia/reperfusion, showed severe tubular cell swelling, interstitial edema, tubular dilatation and moderate to severe necrosis. Hyperlipidemia enhanced renal ischemia/reperfusion injury by elevation of oxidative stress and inflammatory responses.

Key words: Hyperlipidemia, inflammation, ischemia/reperfusion, renal, oxidative stress

INTRODUCTION

Hyperlipidemia is one of major risk factor for cardiovascular disease like atherosclerosis, that may ultimately leads to End Stage Renal Disease (ESRD) (Kumai *et al*., 2003). Hyperlipidemia is at a higher risk of an ischemic condition caused by decreased blood flow (Silvia *et al*., 1999). Thus, ischemia is described as decrease in oxygen supply or increase in oxygen demand. With increasing duration and severity of ischemia, however, greater cell damage can develop, with a predisposition to a spectrum of reperfusion-associated pathologies, collectively called reperfusion injury (Yellon and Baxter, 2000). The recent study demonstrated higher incidence of ischemic nephropathy in hyperlipidemic rat (Anja *et al*., 2004). The mechanisms behind the injury in ischemic nephropathy are not fully understood despite intense research. Hyperlipidemic patients may need renal transplantation in their later life due to ischemic nephropathy. Ischemia/Reperfusion (I/R) injury is one of the dangerous complications of this procedure.

The short period of ischemia (30 min) in hyperlipidemia has been demonstrated to reversible renal failure, leading to progressive injury with ESRD (Silvia *et al*., 1999). Reactive Oxygen Species (ROS) and Nitric Oxide (NO) play an important role in mediating cell damage during I/R injury (Basireddy *et al*., 2006; Noiri *et al*., 2001). Inflammation contributes substantially to the pathogenesis of I/R with a central role for particular cells, adhesion molecules and cytokines (Ysebaert *et al*., 2004). Neutrophils are the inflammatory cells, which produces abundantly ROS during I/R injury. Myeloperoxidase (MPO) is found in neutrophils, which catalyzes the formation of hypochlorous acid (HOCl), a toxic agent to cellular components and initiates oxidative injury (Altunoluk *et al*., 2006). Renal I/R causes tissue injury by oxygen radicals and oxidative stress caused by an imbalance between production of ROS and the antioxidant capacity (Erdogan *et al*., 2006; Yildirim *et al*., 2003). Renal I/R injury may cause oxidative stress and increase lipid peroxidation in the tissue and the tissue of rat decreases antioxidant enzyme activities after renal I/R

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is well reported by Emre *et al*. (2006). Thus aim was pursued in this present study to understand the effect of hyperlipidemia on renal I/R injury.

MATERIALS AND METHODS

Animals and experimental procedures: Male Wistar rats weighting 180-200 g were placed in a quiet and temperature (21±2°C) and humidity (60±5%) controlled room in which a 12-12 h light-dark cycle was maintained. All experiments in this study were performed in accordance with the CPCSEA guideline and were approved by Institutional Animal Ethical Committee (IAEC). Rats were randomly divided into four groups. Group 1 (n = 6): Normal Control (NC), group 2 (n = 6): Hyperlipidemic Control (HC), Group 3 (n = 6): Renal I/R injury (I/R) and group 4 (n = 6): Hyperlipidemic+Renal I/R injury (HC+I/R). Hyperlipidemia was induced by cholesterol (Sigma Chemical Co., St. Louis, MO, USA) feeding in hydrogenated ground nut oil (as a vehicle), was given orally at a dose of 500 mg kg^{-1} for 4 weeks (Kumari *et al*., 2006). At the end of fourth weeks, rats were anesthetized with ketamine (60 mg kg^{-1}, i.p.) and diazepam (5 mg kg^{-1}, i.p.) and renal I/R injury was perform by occlusion of both renal vascular pedicles for 60 min, followed by 24 h reperfusion (Kontogiannis and Burns, 1998).

Biochemical evaluation: At the end of the reperfusion period, blood samples were collected for the measurement of creatinine, urea, Na^+ and lipid profile. Urine samples were collected throughout the reperfusion period and the volume of urine produced recorded. Urine concentrations of Na^+ were measured and used in conjunction with serum Na^+ to estimate fractional excretion of Na^+. All biochemical parameters were measured by semi automated biochemistry analyzer-photometer 5010 (Piramal Healthcare, Mumbai, India) using a commercially diagnostic kits from Piramal healthcare, Mumbai, India.

Kidney tissue (300 mg) was homogenized in ice cold tamponade containing 150 mM KCL for determination of malondialdehyde (MDA), end product of lipid peroxidation, Nitric Oxide (NO), Xanthine Oxidase (XO), reduced glutathione (GSH), catalase, superoxide dismutase (SOD) and myeloperoxidase (MPO) activity were evaluated as a means of oxidative stress. MDA levels were assayed for products of lipid peroxidation. MDA referred to as thiobarbituric acid reactive substance, was measured with thiobarbituric acid at 532 nm as described earlier (Slater and Sawyer, 1971). Reduced glutathione (GSH) was determined by the spectrophotometric method, which was based on the use of Ellman's reagent (Moron *et al*., 1979). Superoxide dismutase (SOD) activity was measure according to method of Misra and Fridovich (1972). Catalase activity was determined according to the method of Aebi (1984), by monitoring the initial rate of disappearance of hydrogen peroxide at the 240 nm in a spectrophotometer.

The Xanthine Oxidase (XO) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm (Prajda and Weber, 1975), where as NO level was estimated by the method of Guevara *et al*. (1998).

Myeloperoxidase (MPO) activity was measured in renal tissues in a procedure similar to that documented by Wei and Frenkel (1993).

Levels of TNF-α in serum were determined by using an Enzyme-Linked Immunosorbent Assay (ELISA) (Endogen, mouse TNF-α kit, Pierce Biotech Int., Rockford, Illinois, USA) according to the manufacturer's instructions.

Histopathological evaluation: After formalin fixation (10% phosphate-buffered) and dehydration, paraffin-embedded renal sections (4 μm) were stained by hematoxylin and eosin. All sections of kidney samples were examined for tubular cell swelling, tubular dilatation, necrosis of epithelium and interstitial edema. Histopathology of renal tissue was evaluated per section in at least 10 randomly selected non-overlapping fields at x100 magnifications of the sections.

Statistical analysis: All the values are expressed as Mean±Standard Deviation (SD). Statistical significance between more than two groups were tested using one-way Analysis of Variance (ANOVA) followed by the Bonferroni's multiple comparisons test using computer based fitting program (Prism, Graphpad 5). Differences were considered to be statistically significant when $p<0.05$.

RESULTS

Lipid profiles: Serum cholesterol, triglyceride and LDL levels were significantly increased and HDL levels were significantly decreased after cholesterol feeding in the HC group when compared to normal control rats (Table 1).

Table 1: Effect of cholesterol diet on serum cholesterol, triglyceride, LDL and HDL levels in rats

Parameters (mg dL^{-1})	NC	HC
Cholesterol	68.7±16.6	204.5±22.47***
Triglyceride	59.8±16.92	212.4±20.19***
LDL	26.4±8.52	136.3±23.87***
HDL	30.4±4.23	25.7±3.34*

Values are represented as a Mean±SD from 6 animals in each group. ***p<0.001, *p<0.05 when compared with normal control group

Table 2: Effect of hyperlipidemia on renal ischemia/reperfusion induced kidney dysfunction in rats

Parameters	NC	HC	I/R	HC+I/R
Serum creatinine (mg dL^{-1})	1.54±0.25	2.18±0.31	4.10±0.46^{a***}	5.12±0.75^{b*}
Serum urea (mg dL^{-1})	60.27±6.90	93.00±8.62	127.90±19.63^{a***}	162.90±31.68^{b*}
Fractional excretion of Na^+ (%)	0.67±0.19	1.50±0.31	15.70±3.55^{a***}	21.19±2.50^{b*}

Values are represented as a Mean±SD from 6 animals in each group. [a]Compared with normal control group, [b]Compared with I/R group, *p<0.05, ***p<0.001

Table 3: Effect of hyperlipidemia in ischemia/reperfusion induced renal oxidative stress and inflammatory response in rats

Parameters	NC	HC	I/R	HC+I/R
MDA (nmole mg^{-1} protein)	1.90±0.30	2.38±0.39	3.63±0.61^{a***}	4.51±0.49^{b*}
SOD (U mg^{-1} protein)	8.78±0.99	8.01±1.36	4.73±0.86^{a***}	3.18±0.53^{b*}
Catalase (U mg^{-1} protein)	7.95±0.86	7.15±0.79	4.28±0.73^{a***}	2.86±0.52^{b*}
GSH (nmole mg^{-1} protein)	20.98±2.51	16.78±3.75	11.57±2.62^{a***}	6.86±1.95^{b*}
XO (U g^{-1} protein)	1.02±0.31	1.18±0.44	1.89±0.37^{a**}	2.50±0.22^{b*}
NO (µmoles g^{-1} tissue)	0.86±0.29	1.34±0.25	1.94±0.40^{a***}	2.83±0.54^{b**}
MPO (mU g^{-1}) protein	31.02±5.75	42.19±10.81	67.49±14.11^{a***}	91.34±12.43^{b*}
TNF-α (pg mL^{-1})	0.75±0.21	1.09±0.27	1.70±0.30^{a***}	2.83±0.50^{b*}

Values are represented as a Mean±SD from 6 animals in each group. [a]Compared with normal control group, [b]Compared with I/R group, *p<0.05, **p<0.01, ***p<0.001

Table 4: Semiquantitative analysis of tubular cell swelling, interstitial edema, tubular dilation and necrosis of epithelium in rats

		Tubular cell swelling				Interstitial edema				Tubular dilatation				Necrosis of epithelium			
Group	N	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
NC	6	6	0	0	0	6	0	0	0	6	0	0	0	6	0	0	0
HC	6	0	5	1	0	0	4	2	0	0	5	1	0	4	2	0	0
I/R[a]	6	0	0	4	2	0	0	5	1	0	0	4	2	0	0	3	3
HC+I/R[b]	6	0	0	0	6	0	0	0	6	0	0	0	6	0	0	1	5

Score 0: No degeneration, 1: Mild degeneration, 2: Moderate degeneration and 3: Severe degeneration. [a]Statistical significant difference from the control group, [b]Statistical significant difference from the I/R group, p<0.05

Kidney dysfunction: When compared to normal control rats, I/R caused a significant increase in serum creatinine and urea levels suggesting marked glomerular dysfunction. HC+I/R rats had significant effect on serum creatinine and urea level when compared to I/R rats, suggesting an enhancement of I/R induced glomerular dysfunction by hyperlipidemia (Table 2).

Fractional excretion of Na^+ was used as an indicator of tubular dysfunction. When compared to normal control rats, I/R caused a significant increase in fractional excretion of Na^+ suggesting marked tubular dysfunction. Hyperlipidemic rat subjected to renal I/R produced significant increase in fractional excretion of Na^+ when compared to I/R rats (Table 2).

Oxidative stress: When compared to normal control rats, I/R caused a significant elevation in MDA content. Hyperlipidemic rat subjected to renal I/R produced significant increase in MDA content when compared to I/R rats. On comparison with normal control rat, renal I/R subjected rat exhibited a significant decrease in antioxidant enzymes like SOD, catalase and GSH. Hyperlipidemic rats subjected to renal I/R shows significant decrease in renal SOD, catalase and GSH level when compared to I/R rats, suggesting exacerbation of I/R induced oxidative stress in hyperlipidemic rat. Hyperlipidemic control rats had no any effect on lipid peroxidation as well as antioxidant enzymes level when compared to normal control rats (Table 3).

The XO enzyme activity, one of the sources of ROS production, was increased in both I/R and HC+I/R groups in comparison with normal control and hyperlipidemic control rats. Hyperlipidemic rat subjected to renal I/R produced significant increase in XO activity when compared to I/R rats. Hyperlipidemia alone did not affect the XO activity (Table 3).

On comparison with normal control rats NO levels were increased in I/R rats. HC+I/R rats had significant rise in NO level when compared to I/R rats. Hyperlipidemia alone did not affect NO level in kidney (Table 3).

Inflammatory response: The MPO activity was used as an indicator of inflammatory response. On comparison with normal control rat, renal I/R subjected rat exhibited a substantial increase in MPO activity, suggesting increased neutrophil infiltration into reperfused renal tissues. Hyperlipidemic rat subjected to renal I/R produced significant increase in MPO activity when compared to I/R rats, where as hyperlipidemia alone had no effect on MPO activity when compared to normal control rats (Table 3).

Renal I/R rats had significantly increased in serum TNF-α level, in comparison to normal control rats. Hyperlipidemic rat subjected to renal I/R produced significant increase in TNF-α level when compared to I/R rats (Table 3).

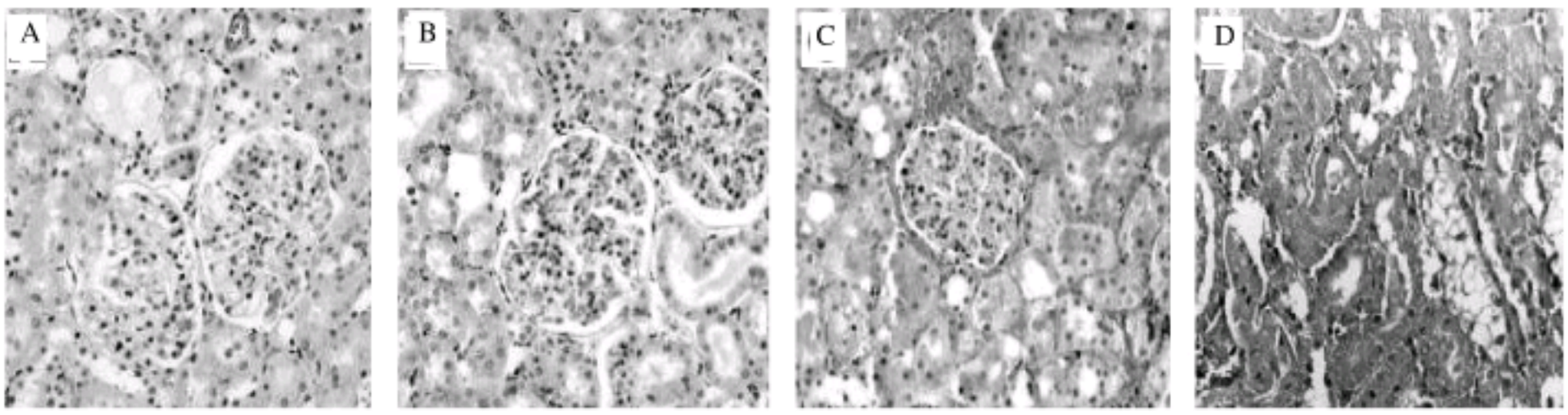

Fig. 1: Kidney morphology in: (A) control rat, (B) rat treated with high cholesterol diet only, (C) rats subjected to renal ischemia/reperfusion injury only and (D) hyperlipidemic rat subjected to renal ischemia/reperfusion injury (H and E X100)

Histopathological changes: Histopathological examination of kidney showed severe and extensive damage in hyperlipidemic rats subjected to renal I/R injury (Fig. 1D). These changes are shown in Table 4. Figure 1A indicates a kidney section of a control rat while Fig. 1B shows representative images of kidneys of hyperlipidemic rats which have mild degenerative changes. Normal rats subjected to renal I/R injury showed moderate degenerative changes (Fig. 1C, Table 4).

DISCUSSION

In the present study, we used 60 min of ischemia, the importance of the ischemic duration is well established and it has been previously shown that a longer period of ischemia causes a more severe injury. The degradation of ATP to hypoxanthine and xanthine via inosine is enhanced with the duration of ischemia (Osswald *et al*., 1977). The restoration of ATP levels during reperfusion is slower after prolonged ischemia (Stromski *et al*., 1986). Temperature is a critical factor in ischemic injury. Hyperthermia, especially during the ischemic phase, leads to a more severe renal I/R injury. Raising the temperature from 37 to 39.5°C during ischemia leads to a 100% increase in Blood Urea Nitrogen (BUN) in a model using 30 min of ischemia and uninephrectomy (Zager, 1990). In order to keep the temperature constant we used a servo controlled heating pad that kept the temperature in the rat at 37.5°. An important question in this study is how hyperlipidemia could cause the increased sensitivity to renal I/R, which observed in hyperlipidemic animals. Several possible explanations exist. The increased sensitivity to I/R could be due to high level of LDL per se. Secondary effects of LDL such as oxidation of LDL, increased oxidative stress, hemodynamic alterations and formation of NO could also be involved.

In present animals subjected to renal I/R demonstrated an increase in serum creatinine and urea level, indicating glomerular dysfunction. Renal I/R also caused a marked increase in fractional excretion of Na^+ indicating marked tubular dysfunction. Moreover, characteristic histological signs such as tubular cell swelling, tubular dilatation, necrosis of epithelium and interstitial edema were observed in kidneys subsequent to renal I/R. Animals subjected to renal I/R demonstrated an increase in the renal MDA levels and attenuated the antioxidant enzyme pool. Renal I/R induced oxidative stress in hyperlipidemia was associated with impaired renal function leading to a marked increase in serum creatinine, BUN, fractional excretion of Na^+ and morphological changes.

Oxidative stress and inflammatory response might play a patophysiological role in renal I/R injury in hyperlipidemia given the knowledge that oxidative stress is implicated both in the complications of hyperlipidemia and renal I/R. Elevated oxidative stress has been demonstrated in renal and liver in hyperlipidemic rats (Hartmut *et al*., 2000; Bolkent *et al*., 2004). The combined oxidative stress from two sources may thus increase the total level of ROS. Infiltration of inflammatory cells is one of the main features of renal I/R injury in hyperlipidemic rats. The infiltrate mainly consisted of cells identified as macrophages/monocytes and T-lymphocytes. The inflammatory response is increased acutely after I/R of the brain in hyperlipidemic animals (Eunhee *et al*., 2008). It is likely that inflammatory cells contribute to increased oxidative stress in hyperlipidemic kidneys after I/R. According to Sakr *et al*. (1992) pretreatment with a single injection of tacrolimus, 24 h prior to 60 min of ischemia was able to decrease the renal injury and the effect was associated with decreased levels of TNF-α. Thus we decided to estimate TNF-α and MPO. In our finding, the

erum level of TNF-α was higher in HC+I/R group, might be a one reason of exaggeration of renal I/R in hyperlipidemia.

The cardiac MPO activity increased after renal I/R, consistently with leukocyte infiltration and activation. The active neutrophils show high MPO activity in the tissue as an inflammatory answer (Kelly, 2003). The present work demonstrated that, the high renal MPO activity after induction of I/R in hyperlipidemic rats, is very important because it clearly shown high leukocyte infiltration in the renal tissue. The neutrophils play a major role in oxidant injury via the mechanisms such as the action of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase or MPO system. Hypochlorous acid is produced largely from stimulated neutrophils by MPO activity. Hypochlorous acid causes oxidation of other molecules such as proteins, amino acids, carbohydrates, nucleic acids and lipids, expanding renal tissue damage (Arnhold *et al*., 2001).

The nitric oxide system may be involved in the increased sensitivity to I/R in hyperlipidemia. There is evidence for increased NO-production in the hyperlipidemic rat (Onody *et al*., 2003). The reaction of NO with O_2^- results in peroxynitrite formation, a potent and aggressive cellular oxidant and causes the formation of 3-nitro-L-tyrosine (Yagmurca *et al*., 2004; Sudnikovich *et al*., 2007). Nitrite/nitrate levels, as the end products of nitric oxide conversion, were increased in cardiac tissue in hyperlipidemic animals comparison with non hyperlipidemic animals (Onody *et al*., 2003), which was confirmed by elevated NO level in present study.

Several mechanisms might be responsible for the exaggerated renal injury in hyperlipidemia. First, the restoration of hyperlipidemia, some previous works supports the importance of plasma cholesterol level in I/R injury. In present study, we found severe renal injury when I/R performed in hyperlipidemic rats, in which cholesterol level was higher than in the normal rats. Hyperlipidemia, the elevated cholesterol level during I/R could be deleterious for the kidney. An increased acute sensitivity to ischemia has been demonstrated when plasma cholesterol level was raised by high cholesterol diet in combination with renal I/R in rats (Silvia *et al*., 1999).

In conclusion, the renal I/R injury caused renal damage via oxidative stress and inflammatory process in normal rats. Also, the results showed that hyperlipidemia enhanced renal damage induced by I/R in hyperlipidemic rats by the way of increasing lipid peroxidation and decreased antioxidant enzyme activities as well as increasing inflammatory response.

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