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# Severely modified lipoprotein properties without a change in cholesteryl ester transfer protein activity in patients with acute renal failure secondary to Hantaan virus infection

*Jihoe Kim*[1,#], *Hyun Ho Park*[1,#], *Inho Choi*[1], *Young Ok Kim*[2] *& Kyung-Hyun Cho*[1,*]

[1]School of Biotechnology, Yeungnam University, Gyeongsan 712-749, [2]Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul, Korea

**Patients with hemorrhagic fever with renal syndrome (HFRS) often exhibit altered serum lipid and lipoprotein profile during the oliguric phase of the disease. Serum lipid and lipoprotein profiles were assessed during the oliguric and recovery phases in six male patients with HFRS. In the oliguric phase of HFRS, the apolipoprotein (apo) C-III content in high-density lipoproteins (HDL) was elevated, whereas the apoA-I content was lowered. The level of expression and activity of antioxidant enzymes were severely reduced during the oliguric phase, while the cholesteryl ester transfer protein activity and protein level were unchanged between the phases. In the oliguric phase, electromobility of $HDL_2$ and $HDL_3$ was faster than in the recovery phase. Low-density lipoprotein (LDL) particle size was smaller and the distribution was less homogeneous. Patients with HFRS in the oliguric phase had severely modified lipoproteins in composition and metabolism. [BMB reports 2010; 43(8): 535-540]**

## INTRODUCTION

Hemorrhagic fever with renal syndrome (HFRS) is caused by infection with the Hantaan virus (Family *Bunyaviridae*), which is broadly distributed throughout Europe, Russia, and Asia (1). The principal symptoms of HFRS include fever and hemorrhagic manifestations, such as petechiae and acute renal impairment. Patients with HFRS develop acute renal failure complicated by proteinuria, hematuria, hyponatremia, and hyperphosphatemia (2, 3). Acute renal failure, as well as chronic kidney disease, is associated with hypertriglyceridemia and a reduction in high density lipoprotein-cholesterol (HDL-C) (4). The lipid metabolism disorders are accompanied by significant and transient changes in the levels of expression of apolipoprotein (apo), specifically apoC-I and apoC-III (5). Changes in the levels of lipoproteins/apolipoproteins are linked to the incidence of cardiovascular disease (CVD). Adults with chronic kidney disease also have a high burden of CVD (6). Indeed, CVD is a frequent cause of death in patients on chronic hemodialysis (7).

Thus, we have determined the changes in lipoprotein metabolism occurring in patients in the oliguric phase of HFRS. In the current study, the lipoprotein profiles of six male patients with HFRS who were admitted to the Nephrology Unit of Uijeongbu St. Mary's Hospital (Uijeongbu, Korea) were analyzed. We determined the lipoprotein metabolism parameters from the sera of patients with HFRS in the oliguric phase, as well as sera from the same patients after recovery. Four normolipidemic serum samples from age- and gender-matched healthy persons were also analyzed and compared to the serum samples from the patients with HFRS.

## RESULTS

### Hypertriglyceridemic serum lipid profile in the oliguric phase

In the oliguric phase, blood urea nitrogen (BUN) and creatinine (Cr) levels were increased by 5.4- and 7-fold, respectively, as compared to the recovery phase levels, as shown in Table 1. The calculated glomerular filtration rate (GFR) was decreased in the oliguric phase (12.5 ± 3.7 ml/min), but increased (71.7 ± 23.1 ml/min) in the recovery phase.

In the oliguric phase, the serum cholesterol concentrations were significantly reduced by approximately 32%, and the serum triglyceride (TG) concentrations were increased significantly (up to approximately 203% of the recovery phase levels), indicating a change in the hypocholesterolemic and hypertriglyceridemic lipid profiles. The average HDL-C level was reduced in the oliguric phase. After recovery, the HDL-C reverted back to normal levels, similar to that of the reference levels. Interestingly, serum total protein and albumin concentrations were reduced by approximately 20% and 30% in the oliguric phase as compared to the recovery phase, respecti-

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[*] Corresponding author. Tel: 82-53-810-3026; Fax: 82-53-814-3026; E-mail: chok@yu.ac.kr
[#] These authors contributed equally to this work.

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**Table 1.** Serum parameters and enzyme activities obtained from patients with hemorrhagic fever and renal syndrome during oliguric and recovery phase as compared to healthy controls*

| | Oliguric (n = 6) | Recovery (n = 6) | Reference (n = 4) |
|---|---|---|---|
| Age (yr) | 40.8 ± 3.8 | 40.8 ± 3.8 | 42 ± 4.5 |
| BMI ($kg/m^2$) | 25.5 ± 0.8 | 25.8 ± 1.1 | 24.5 ± 1.0 |
| TC (mg/dl) | 146 ± 16$^{\ddagger}$ | 214 ± 39 | 189 ± 20 |
| TG (mg/dl) | 403 ± 167$^{\S}$ | 198 ± 62 | 148 ± 22 |
| HDL-C (mg/dl) | 29 ± 2$^{\S}$ | 39 ± 6 | 41 ± 4 |
| BUN (mg/dl) | 82 ± 23$^{\S}$ | 15 ± 3 | 11± 5 |
| Total bilirubin (mg/dl) | 0.53 ± 0.2 | 0.58 ± 0.1 | 0.2-1.2$^{\dagger}$ |
| Albumin (g/dl) | 3.0 ± 0.2$^{\ddagger}$ | 4.3 ± 0.3 | 4.5 ± 0.4 |
| GOT (U/L) | 81 ± 34$^{\S}$ | 23 ± 14 | 20 ± 8 |
| GPT (U/L) | 27 ± 9$^{\ddagger}$ | 10 ± 5 | 18 ± 3 |
| LCAT (% CE-conversion/hr/50 μl of serum) | 1.6 ± 0.2$^{\S}$ | 8.5 ± 0.2 | 10.6 ± 1.5 |
| PON (μU/L/10 ml of serum) | 30 ± 5$^{\S}$ | 73 ± 9 | 76 ± 6 |
| CETP (% CE transfer/50 μl of serum) | 27 ± 3 | 24 ± 2 | 21 ± 3 |

BMI: body mass index, BUN: blood urea nitrogen, CETP: cholesteryl ester transfer protein, GOT: glutamic oxaloacetic transaminase, GPT: gamma-glutamic pyruvic transaminase, HDL-C: high-density lipoprotein-cholesterol TG: triglyceride, *Data are expressed as the mean ± SD from three independent measurements, $^{\dagger}$Normal range from guidelines, $^{\ddagger}$P < 0.05 versus recovery phase (paired t-test) $^{\S}$P < 0.01 versus recovery phase (paired t-test).

vely. The serum glutamic oxaloacetic transaminase (GOT) and gamma-glutamic pyruvic transaminase (GPT) values in the oliguric phase were lowered and they were lowered to the normal range in recovery phase. The serum total bilirubin level was unchanged between the oliguric and recovery phases. In general, the serum parameters of lipid and protein were similar with the control values in the recovery phase, as shown in Table 1.

## Immunodetection

SDS-PAGE analysis with equal amounts of $HDL_3$ (5 g/lane) revealed that the composition of apolipoproteins were markedly changed between the phases (supplemental figure). Corresponding to the molecular weight, apoA-I (28 kDa) and apoA-II (17 kDa) are major proteins observed in both the oliguric and recovery phases. ApoC-III band (14 kDa) appeared distinctly in the oliguric phase as indicated by the rectangular box (supplemental figure). Immunodetection revealed that the apoC-III level was elevated only in the oliguric phase (Fig. 1). In the same period, the level of expression of apoA-I was reduced in the oliguric phase by approximately 2-3-fold, as compared to of the recovery phase samples based on immunodetection (Fig. 1). This result indicates a different expression of apolipoproteins in the oliguric phase of HFRS in that apoA-I was decreased and apoC-III was increased.

## Electrophoretic migration of HDL

The purified HDL from each patient was compared via electrophoresis using agarose gel, as the migration ability of each lipoprotein is highly dependent on the intact charge and size. $HDL_2$ from the oliguric phase migrated faster than in the recovery phase, as shown by agarose gel electrophoresis with equal loads of protein (5 μg in each lane), thereby indicating that the $HDL_2$ of t he oliguric phase may have smaller particles and a greater charge, and after recovery the size and charge are restored (Fig. 2A).

This result correlates well with our previous report (8) that HDL in the oliguric phase may have a decreased particle size and/or increased negative charge with down-regulated protein levels.

## ELISA-based detection of apoC-III

Sandwich ELISA-based detection revealed that the serum apoC-III level from the oliguric phase was approximately 3-fold higher than the recovery phase (Table 2), which fell in the normal range (123±32 μg/ml). All lipoprotein fractions showed significantly elevated apoC-III levels in the oliguric phase. ApoC-III in very low density lipoprotein (VLDL) from the oliguric phase was 4.5-fold higher than the recovery phase. $HDL_2$ and $HDL_3$ from the oliguric phase were 3.6- and 3.1-fold higher than the apoC-III level of the recovery phase, respectively (Table 2).

## Activities of HDL-associated enzymes

Lecithin:cholesterol acyltransferase (LCAT) activity was almost deprived in the oliguric phase, as shown in Table 1. The cholesteryl ester (CE)-conversion activity was 82% lower in the oliguric phase compared to the recovery phase. Immunodetection revealed that LCAT band was undetectable in the oliguric phase, and was restored back to normal levels (similar to the control sera), indicating that LCAT activity and expression were lost in the oliguric phase (Fig. 1).

Paraoxonase (PON) activity was reduced approximately 60% in the oliguric phase compared to the recovery phase

(Table 1). The level of protein expression of PON in the oliguric phase was reduced, and increased back to control levels on recovery, as shown in Fig. 1. These results show that LCAT and PON, which are crucial in maintaining antioxidant activity of HDL, were transiently impaired with respect the activity of expression in the oliguric phase.

## CETP activity was not changed in the oliguric phase

Cholesteryl ester transfer protein (CETP) activity and the level of immunodetected protein were not changed between the oliguric and recovery phases, as shown in Table 1 and Fig. 1. The CE-transfer activity to low density lipoprotein (LDL) from [3H]-CE-HDL was 23 ± 3% in the oliguric phase and 24 ± 2% after recovery, while the control sera had levels of 21 ± 2%.

## Change of LDL properties

The particle size of LDL and its distribution was determined via electron microscopy with the same amount of LDL (0.3 mg/ml of protein). Interestingly, LDL from the oliguric phase had a severely distorted morphology, fewer particle numbers and a smaller size (average range of diameter of 46-57 nm). In contrast, LDL from the recovery phase showed a more spherical shape and larger particle size (average diameter range of 65-73 nm; Fig. 2B). These results indicate that normal LDL was prone to change into smaller LDL in the oliguric phase, which

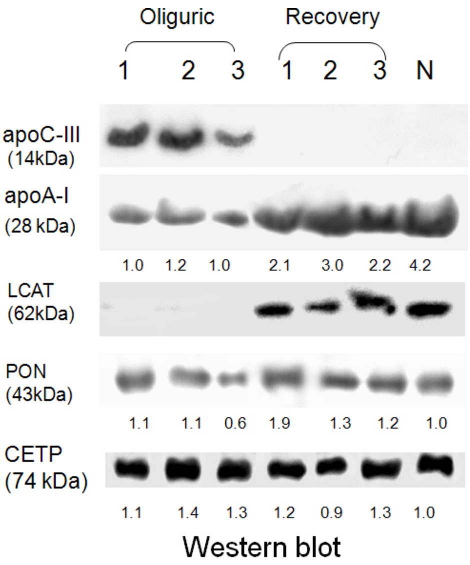

**Fig. 1.** Level of expression of apolipoproteins and enzymes in HDL₃ from representative patients. Equal amounts of HDL₃ (6 μg/lane) were loaded and immunodetected with anti-apoC-III (#AB821; Chemicon) anti-apoA-I (#ab7613; Abcam), anti-LCAT Abcam #ab786; Abcam), anti-PON 1 (ab24261; Abcam), and anti-CETP (abcam 19012; Abcam) antibodies. The lower number indicates the relative band intensity of apoA-I in the recovery phase as compared to each of the oliguric phase samples.

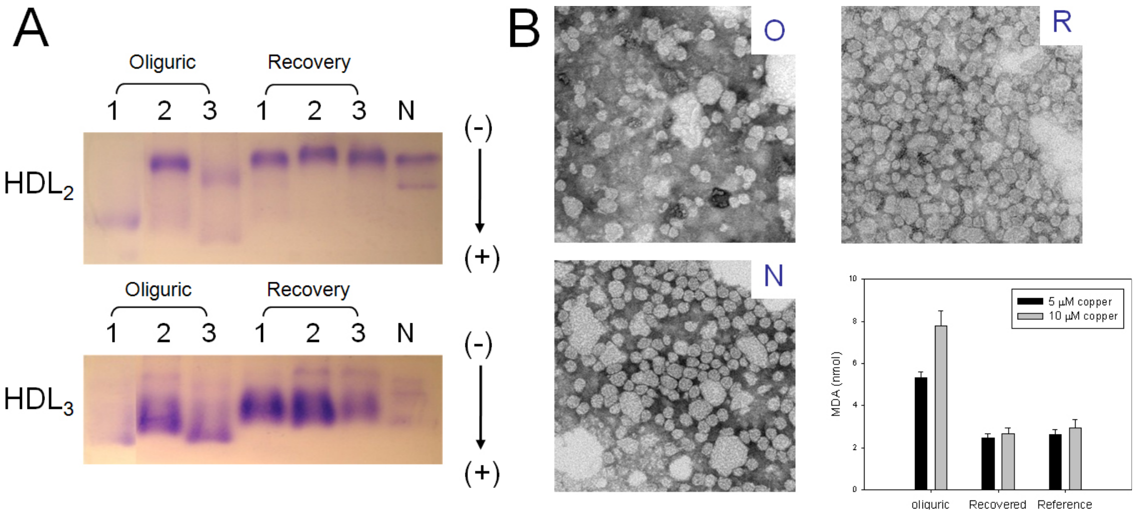

**Fig. 2.** Lipoproteins from the oliguric phase were more decreased in size and more oxidized. (A) Electrophoretic mobility of HDL for comparisons of native particle charge and size. The same amount of proteins (5 μg of protein/lane) were loaded onto 0.7% agarose gels and visualized via Coomassie blue R-250 staining. Lane N, normal subject as reference. Lanes 1, 2, and 3 indicate representative patients from each phase. (B) Representative electron micrograph of LDL from the oliguric and recovery phases. The LDL was negatively stained and all micrographs are shown at a magnification of 30,000x. The scale bar corresponds to 100 nm. LDL from the oliguric phase showed a reduced particle size and a distorted shape, with a heterogeneous population. The inset graph shows susceptibility of LDL-oxidation via copper-mediated oxidation. The same amount of each LDL (108 μg/ml) in PBS (pH 7.4) was incubated for 4 hours at 37°C with cupric ion. The oxidized product (malondialdehyde) was quantified via the thiobarbituric acid reactive substance method. LDL from oliguric sera were twice as sensitive to oxidation as the reference sera. Error bars indicate SD from three independent measurements with duplicate samples.

**Table 2.** Sandwich ELISA-based detection of apoC-III in serum and lipoprotein[*,†]

| | Oliguric (n = 6) | Recovery (n = 6) | Reference (n = 4) |
|---|---|---|---|
| Serum (μg/ml) | 344 ± 55[‡] | 123 ± 32 | 95 ± 26 |
| VLDL (μg/mg of protein) | 147 ± 23[‡] | 33 ± 7 | 20 ± 5 |
| LDL (μg/mg of protein) | 9 ± 5 | nd | nd |
| HDL$_2$ (μg/mg of protein) | 326 ± 48[‡] | 90 ± 16 | 70 ± 21 |
| HDL$_3$ (μg/mg of protein) | 93 ± 23[‡] | 30 ± 7 | 24 ± 6 |

nd: not detected, *Fifty microliters of individual lipoprotein (2 mg of protein/ml) was reacted with apoC-III antibody (AB821), which was coated on a 96-well plate, [†]Data are expressed as the mean ± SD from three independent measurements, [‡]$P < 0.05$ versus recovery phase (paired t-test).

was more susceptible to oxidation in the early stages of viral infection.

LDL from the oliguric phase was more sensitive to cupric ion-mediated oxidation, with nearly 2-fold malondialdehyde (MDA) production by 5 and 10 μM treatment (Fig. 2B). This result suggests that LDL in the oliguric phase was more prone to oxidation in the presence of an equal pro-oxidant concentration, thereby indicating that oxidative stress in the oliguric phase might be higher than the normal phase.

## DISCUSSION

In the current study, changes in the composition, function, and structure of lipoproteins between the oliguric and recovery phases of HFRS were evaluated, particularly with respect to apolipoprotein composition and the enzymatic activity of HDL-C. Although the number of patients was relatively small in this study (due to strict inclusion criteria), we provided more detailed evidence for functional and structural changes in the lipoprotein levels, including severe deprivation of the HDL-associated enzymes, LCAT and PON. Physiologically, the serum bilirubin levels did not change between the oliguric and recovery phases. This phenomenon is comparable to the symptoms of scrub typhus, or tsutsugamushi, in which total bilirubin levels are elevated (9), thereby indicating that abnormal liver function occurs only infrequently in patients with HFRS.

The current results confirmed that the increased apoC-III is strongly associated with hypertriglyceridemia (Tables 1 and 2). It is known that apoC-III inhibits lipolysis of VLDL via inhibition of LPL and hepatic triglyceride lipase (HTGL; 10). The plasma concentrations of apoC-III and TG are positively correlated with the development of metabolic syndrome (11). The amount of apoC-III in apo-B containing lipoprotein has been identified as an independent risk factor for CHD (12). In addition, an increase of apoC-III content in HDL resulted in changes in several HDL properties, including decreased particle size, phospholipid content, and a decreased number of apoA-I molecules (13).

Furthermore, apoC-III in VLDL and LDL has been shown to be a significant predictor of coronary events (14). Excessive apoC-III may contribute to the atherogenicity of VLDL particles, as apoC-III inhibits VLDL lipolysis (15), uptake, and clearance from the plasma by normal, high-affinity receptors on the hepatocytes (16). Recently, LCAT-deficient patients (-/-homozygote) have been shown to have a severe decrease in apoA-I and apoA-II (17), with relatively increased serum TG concentrations, thereby indicating that LCAT activity is correlated with the expression of apolipoproteins and a hypertriglyceridemic lipid profile. There may exist a negative linkage between apoC-III levels and LCAT activity, as well as apoA-I. Taken together, the malfunction of HDL in the oliguric phase can be explained by the fact that LCAT performs a crucial function in the maturation of HDL particles (18).

Interestingly, serum CETP activity and protein levels were not reduced in the oliguric phase of HFRS (Fig. 1), although LCAT and PON activities were significantly reduced. This result may help to explain why serum TG accumulated in VLDL; HDL-TG and LDL-TG may be transported to the VLDL via CETP. The depletion of TG in HDL and VLDL may be connected to a decrease in particle size, as shown in a previous report (8) and the current report (Fig. 2). This result is also in good agreement with the recent report of Jahangiri *et al*. (19) that showed CE-transfer activity was maintained despite the striking alterations in HDL composition during acute phase.

The current results demonstrated that LDL from the oliguric phase was smaller than the reference LDL with more sensitivity to cupric ion-mediated oxidation (Fig. 2), supporting the notion that the oliguric-phase LDL was more prone to oxidization. The shift to greater atherogenic lipoprotein predominance could be the result of alterations in the properties of HDL (20), as well as LDL. This result correlates well with the smaller LDL size and faster electromobility in the oliguric phase than the recovery phase.

In conclusion, abnormal lipoprotein parameters in the oliguric phase of HFRS appeared to be quite similar to those of patients with chronic hemodialysis and viral infections, i.e., hypocholesterolemic and hypertriglyceridemic serum profiles with reduced HDL particle size and cholesterol levels, and apoC-III overexpression in the HDL. Serum LCAT and PON activity were significantly reduced in the oliguric phase, with reductions in HDL-C and particle size, while CETP activity was not changed between the phases.

## MATERIALS AND METHODS

### Blood sampling

Sera were obtained from patients (mean age = 40.8 ± 3.8 years) during the oliguric phase of HFRS (n = 6, body mass index[ BMI] = 25.5 ± 0.8 kg/m$^2$) and after recovery 30-40 days after the oliguric phase (n = 6, BMI = 25.8 ± 1.1 kg/m$^2$). None of the patients with HFRS took medications during the oliguric phase, nor did any of the patients drink alcohol excessively before or after the oliguric phase. The patients had no histories of endocrinologic disorders, including hyperlipidemia, diabetes mellitus, or hypertension. Gender- and age-matched reference sera were obtained from four healthy volunteers (mean age = 42 ± 4.5 years; BMI = 24.5 ± 1.0). All of the reference individuals were healthy and had unremarkable medical histories. Informed consent was obtained from the patients and the reference subjects, and the protocols of this study were approved by the Institutional Review Board of the Catholic University of Korea (Seoul, Korea).

### Isolation of lipoproteins

VLDL ($1.006 < d < 1.019$ g/ml), LDL ($1.019 < d < 1.063$), HDL2 ($1.063 < d < 1.125$), and HDL3 ($1.125 < d < 1.210$) were isolated from the sera via sequential ultracentrifugation, in accordance with the method described by Havel *et al.* (21), at 100,000 x g for 22 hours at 10°C using a Himac CP-90$\alpha$ (Hitachi, Tokyo, Japan).

### Determination of serum lipids and proteins

Serum parameters, lipids, and glucose concentrations were determined using an automatic blood analyzer (Fuji DRI-CHEM, FDC-3000; Tokyo, Japan). Protein concentrations in lipoprotein species were determined via the Lowry protein assay, as modified by Markwell *et al.* (22) and using the Bradford assay reagent (BioRad, Seoul, Korea) with bovine serum albumin as a standard.

### Calculation of GFR

The GFR was calculated using the Cockcroft-Gault formula (23) as follows: GFR = [(140 − age) x body weight] / (72 x serum creatinine).

### HDL-associated enzyme assay

LCAT, CETP, and PON assays were carried out as described in the supplemental materials, as previously reported (24-26).

### Enzyme linked immunosorbent assay (ELISA)

For the sandwich ELISA, diluted primary antibody (1 : 2,000 or 1 : 4,000) was coated onto a 96-well plate (Nunc Maxisorp #439454) overnight at 4°C. As primary antibodies, apoA-I (#ab7613; Abcam, Cambridge, UK) and apoC-III were obtained (#AB821; Chemicon, Temecula, CA, USA). Horseradish peroxidase (HRP)-conjugated apoA-I antibody (ab20784) and HRP-apoC-III antibody (ab27624) were purchased from Abcam and used as secondary antibodies. A substrate reagent pack (DY999; R&D Systems, Minneapolis, MN, USA) was used for color development. Human apoC-III was purchased from Chemicon (#ALP60) to use as a concentration standard with serial dilution. All serum samples (1 : 500 diluted equally), and lipoprotein fractions (2 mg of protein/ml) were incubated with 50 μl/well. The plate was washed three times using PBS containing 0.5% (v/v) Tween-20 (PBST).

### Electrophoresis and Western blot analysis

The apolipoprotein/lipoprotein compositions were compared via SDS-PAGE with the same amount of protein loading, and the level of expression of apolipoprotein was analyzed by immunodetection using anti-apoC-III (#AB821; Chemicon), anti-apoA-I (#ab7613; Abcam), anti-LCAT (#ab786; Abcam), anti-PON 1 (#ab24261; Abcam), and anti-CETP antibodies (#ab 19012; Abcam). The relative band intensities were compared via band scanning using a Gel Doc® XR (Bio-Rad, Hercules, CA, USA) with Quantity One software, version 4.5.2.

### Electromobility of lipoproteins

The migration of each lipoprotein is dependent on its intact charge and size of particle, and was compared via electrophoresis in agarose gels in native conditions (27). The gels were dried and stained with 0.125% Coomassie brilliant blue in order to visualize the lipoprotein bands.

### Susceptibility of LDL oxidation

In order to evaluate susceptibility via copper-mediated LDL-oxidation, each diluted LDL (108 μg/ml) in phosphate buffered saline (PBS; pH 7.4) was incubated for 4 hours at 37°C with different concentrations of $CuSO_4$. After incubation, the degree of LDL-oxidation was determined via measurements of the quantities of thiobarbituric acid reactive substances (TBARS) generated at 517 nm (28).

### Electron microscopy

Transmission electron microscopy (TEM) was performed with a Hitachi electron microscope (Ibaraki, Japan), model H-7600, operating at 80 kV. Each LDL was negatively stained with 1% sodium phosphotungastate (PTA; pH 7.4) at a final protein concentration of 0.3 mg/ml in PBS. Five μl of the LDL suspension was blotted with filter paper and replaced immediately with a 5 μl droplet of 1% PTA. After a few seconds, the stained LDL fraction was blotted onto a Formvar carbon-coated 300 mesh copper grid and air-dried. The shape and size of the LDL were determined via TEM photography at a magnification of 30,000x.

### Statistical analysis

All values were expressed as the mean ± standard deviation (SD), and changes between the oliguric and recovery phases were evaluated by a paired t-test using SPSS (version 14.0;

SPSS, Inc., Chicago, IL, USA). Statistical significance was defined as a $P < 0.05$.

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