Impairment of Renal Function and Electrolyte Balance in Rabbit Hemorrhagic Disease

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(Received 11 July 2007/Accepted 8 May 2008)

ABSTRACT. Rabbit hemorrhagic disease virus (RHDV) induced viral fulminant hepatitis in adult rabbits. We investigated the damage of renal function and electrolyte balance in experimentally infected rabbit by measuring the related serum parameters to elucidate the pathogenesis of RHDV as an index for medical treatment. Nineteen New Zealand White rabbits, ten females and nine males, were each intramuscularly inoculated with 0.5 ml 50% rabbit lethal dose (RLD₅₀) rabbit hemorrhagic disease virus. Blood samples were collected at 0 hr post inoculation (HPI) and every 6 hr from 18 HPI repeatedly through 66 HPI. After virus inoculation, serum blood urea nitrogen (BUN), creatinine (CREA) and sodium (Na⁺) were elevated to a highly significant level (p<0.0001), whereas serum potassium (K⁺) was moderately elevated to a significant level (p<0.05). Hypoglycemia developed highly significantly (p<0.0001). Serum chloride ion (Cl⁻) was the only parameter which did not change significantly (p=0.077). No significant sexual difference was observed among these parameters. Renal insufficiency progressed from 36 hr, as indicated by the increases in BUN and CREA; significant changes in electrolytes resulting in the increased osmolality of extracellular fluid that induced flow disturbance which consequently destroy the homeostasis in cells. Therefore, the later impairments in renal function and electrolyte balance might be an important threat for rabbits which might have survived from acute fulminant hepatitis in RHD.

KEY WORDS: electrolyte balance, rabbit hemorrhagic disease virus, renal insufficiency, serum.

Rabbit hemorrhagic disease (RHD) was first reported in 1984 in the People’s Republic of China [12] and widespread worldwide to Europe, Australia, Mexico, North Africa and New Zealand in the following years. In 1994, RHD was first recognized in Taiwan by Dr. Y. S. Lu through serological tests and was characterized by hemagglutination, electron microscopic examination, viral protein analysis and RT-PCR in 1998 [20]. The characteristic pathological signs for RHD were hemorraghes in the respiratory system, liver, spleen, cardiac muscle, and occasionally in the kidneys. Incubation period in rabbits is 2 to 3 days, morbidity approaches 100%, and mortality is sometimes over 90% in adults. The high morbidty and mortality rates have made RHD a big threat for rabbit industry and also a biological control tool for wild rabbit population. The causative virus, rabbit hemorrhagic disease virus (RHDV), was classified into the family Caliciviridae. This single-stranded non-Envelope RNA virus has a 7.5-kb genome and a 2.2 kb sub-genome well packaged in a 27 to 35 nm (in diameter) icosahedral capsid with a major structural protein of about 60 kDa (VP60), and 10 peripheral cup-shaped depressions[12, 20]. The ability to agglutinate human erythrocytes of group O, A, B and AB were reported [12, 15, 20].

Detection by reverse transcriptase-polymerase chain reaction (RT-PCR) in a rabbit inoculation model showed that the RHDV RNA was present as early as 18 hr post inoculation (HPI) in the liver and spleen, whereas thymus, kidney, tonsil and lymph node were found to be positive after more than 26 HPI [21] or 36 HPI [9]. Liver was considered to be the major organ for RHDV replication [8, 10]. Changes of liver related serum parameters had been studied in several laboratories [6, 19, 23] with suggestion that these parameters are good markers for liver change. However, changes in renal function and electrolytes balance have not been well studied.

After RHDV infection, the glomerular mesangial cells were depleted, and the intracapillary fibrin occlusion characteristic of disseminated intravascular coagulation (DIC) produced severe renal damage [2]. The systemic circulatory dysfunction associated with necrotizing hepatitis and DIC could result in the death of rabbit [17]. Kidney plays an important role in the elimination and conservation of many chemical components of the blood. Renal dysfunction may alter blood homeostasis and then caused advanced renal disease. Hence, the impairment of renal function and the imbalanced electrolyte also played important roles in mortality. The RHDV infection consistently reproduces clinical, biochemical, and histological features of the fulminant liver failure syndrome and thus satisfies the criteria for a suitable animal model [23] for studies associated with human hepatitis research. The purpose of this study was to investigate the damage of renal functions and electrolyte balance by measuring related serum parameters for elucidating the pathogenesis of RHDV as an index for medical treatment.

MATERIALS AND METHODS

Animals: Nineteen (9 males and 10 females) 15-week-old clinically healthy young New Zealand White rabbits, weigh-
ing 2.69 ± 0.16 kg, were obtained from National Animal Health Research Institute in Taiwan. The rabbits were kept in sanitized wired cages and separated by sex. All were fed with commercial dry rabbit feed (Fwu-Sow Industry Co., Taichung, Taiwan, “Laboratory Rabbit” feed) with 19% crude protein, 10% crude fiber and 2500 kcal/kg metabolic energy. None of the rabbits were previously vaccinated with RHD-killed vaccine. The rabbits were kept and handled according to the regulations laid down by IACUC (Institutional Animal Care and Use Committee) of NCHU (National Chung Hsing University).

**Virus and inoculation:** The RHDV was produced from homogenized liver specimen of a field case, and a 10% suspension was made in 0.01 M phosphate-buffered saline. The fluid was centrifuged at 7,000 × g for 30 min at 4°C and then filtered through 0.22 μm filter. The virus suspension was diluted in minimal essential medium into 10^{-4} to 10^{-9} concentration to titrate its infectivity in rabbit, 50% rabbit lethal dose (RLD_{50}), by Reed-Muench method. After the titration, the experimental subjects were each inoculated intramuscularly with 0.5 ml RHDV suspension containing 1 RLD_{50}.

**Pathologic examination:** Six randomly selected rabbits were sacrificed by electronic stunning at different hr post inoculation (HPI) for pathologic examination, including 2 at 18 HPI, 2 at 24 HPI, 1 at 30 HPI and 36 HPI, respectively. All the kidneys and livers were fixed in 10% buffered formalin for 24 hr, processed and embedded in paraffin. Sections of 4 μm-thin were cut and stained with hematoxylin and eosin for light microscopic examination.

**Blood sample collection:** 2.5 ml of blood were obtained from auricular artery at 0 HPI and every 6 hr from 18 HPI repeatedly until rabbits died or been sacrificed. Samples in syringes without anticoagulant were left at room temperature for 30 min, and then at 4°C for 30 min for serum collection. Crude sera were then centrifuged at 1,000 × g for 10 min, and the liquid fraction was harvested for measurements of serum parameters.

**Serum parameter tests:** For renal functions, serum blood urea nitrogen (BUN), creatinine (CREA) and glucose were measured by a spectrophotometric autoanalyzer (7150, Hitachi Co., Tokyo, Japan). The electrolyte concentrations of serum parameters. The electrolyte concentrations were measured by CIBA CORNING Electrolyte 644 autoanalyzer (Ciba Corning Co., MA, U.S.A.).

**Data analysis:** Data from both sexes at different HPI were analyzed by Statistic Analysis System (SAS 9.1). The means and standard deviations were calculated by Means Procedures, and the Least Squares Means of General Linear Model (GLM) Procedure was applied for the analysis of the significant differences [5] on the effect of time and sex. P values less than 0.05 were considered significant, and <0.0001 were considered highly significant. The analysis for frequency distribution was then applied for the interpretation of the inner changing tendency. The strength of the relationship among the parameters was defined by the Pearson correlation coefficient (r) and its’ significance by Correlation Procedure.

**RESULTS**

**Pathologic findings:** Ten rabbits (77% in 13 rabbits) died within 46 hr after RHDV inoculation. Rabbits that died acutely before 30 HPI showed minor renal lesion and insignificant change in BUN and/or CREA values. Diffuse pinpoint hemorrhagic spots on kidneys of dead or sacrificed rabbits at 30, 37 and 46 HPI were observed. “Turkey egg” appeared at 34 HPI and 58 HPI. Congestion, mild to moderate cloudy swelling and tubular degeneration were found through the experimented period. Various degrees of cloudy swelling and fibrin deposition were present in the kidneys. Microthrombi in glomeruli could sometimes be seen, and acidophilic bodies of hyaline cast were identified. Microthrombi that accumulated in small blood vessels were also noted. Rabbits that died after 36 HPI had typical DIC lesion. Figure 1 shows the microscopic findings of kidneys and the related BUN, CREA, K^+, Cl–, glucose and values. Figure 4 shows the progressive changes in microscopic findings of livers. The necrosis of the hepatocytes spread predominantly from the portal trial, while massive or submassive hepatic necrosis was seen in all liver areas, mainly at the periphery of the lobules. Figure 4A demonstrated the degeneration and necrosis of hepatocytes and congestion at portal area. Fatty change at portal area was observed at 37 HPI (Fig. 4B). Massive necrosis close to central vein appeared at 45 HPI (Fig. 4C). Mononuclear cell infiltration at portal area was found in the liver of the rabbit that be sacrificed at 66 HPI (Fig. 4D).

**BUN and CREA:** The BUN and CREA increased significantly and elevated with similar trend (Figs. 2A, 2B). Significant elevation (p<0.05 vs. baseline) appeared at 36 HPI for BUN and 42 HPI for CREA. Frequency distribution revealed that the time which 100% rabbits had values over the maximal value of 0HPI (normal value) were 36 HPI for BUN and 42 HPI for CREA (Figs. 3A, 3B). In comparison with the means at baseline (0 HPI), the peak increment was 3.4 folds higher at 66 HPI for BUN and 2.9 folds at 54 HPI for CREA. Although the means of BUN and CREA were higher than the baseline in rabbits prior to death, the surviving rabbits (survived over 58 HPI) had even higher means prior to sacrifice (Table 1). The BUN/CREA ratio also elevated significantly with peak increment at 60 HPI (Fig. 2C). No sexual difference was observed.

**Glucose:** Serum glucose concentration diminished from 24 HPI (Fig. 2E). The significant decreases (vs. baseline values) were appeared at 30 HPI and 42 HPI. Rabbits with serum glucose concentration below 50 mg/dl were distributed mainly from 24 HPI to 42 HPI which coincided with the major period of death (Fig. 3C). The rabbits would die within 2 hr when the glucose level was lower than 20 mg/dl (in 5 rabbits). The dying rabbits had significantly lower values than the surviving ones (Table 1). However, if the glucose concentration was restored later, the rabbit might survive. For the 3 rabbits that survived 48 hr, their lowest
glucose values appeared at 36 HPI (26.5 mg/dl, 54.2 mg/dl and 57.4 mg/dl) and then restored to normal range from 42 HPI through the end of the experiment. No sexual difference was observed.

**Electrolytes:** For electrolytes, changes in Na\(^+\) and K\(^+\) reached significant level, whereas the change in Cl\(^-\) was insignificant (Figs. 2F, 2G, 2H). Frequency distribution indicated that the percentage of rabbits with serum Na\(^+\) over 145 mEq/l increased at 30 through 54 HPI (Fig. 3C). Hypokalemia (value lower than the normal range: 3.45 mEq/l) developed first at 18 HPI until 24 HPI (in 95% and 58% rabbits, respectively). Then hyperkalemia developed (serum levels higher than the normal range: 4.47 mEq/l) from 30 HPI through 48HPI (Figs. 2G, 3C) which was the period that massive mortality occurred. The 10 rabbits that died had significantly higher mean Na\(^+\) and K\(^+\) levels than the surviving ones (Table 1).

Osmolality was conventionally calculated using the formula mOsm/kg=1.86 [Na\(^+\)+K\(^+\) (mmol/l)] + [glucose (mg/dl)+18] + [BUN (mg/dl)+2.8] in samples with either increased glucose or BUN concentration [11]. After RHDV infection, the osmolality elevated constantly from 18 HPI through the end of the experiment. Significant hyperosmolality appeared at 36 HPI (Fig. 2D) with Osmolal gap values > 20 mOsm/kg, and peaked at 54 HPI with Osmolal gap values > 27 mOsm/kg. The mean of surviving rabbits was higher than the dead ones (Table 1).

**Correlation coefficient analysis among parameters:** Among all parameters measured in this study, only CREA values significantly and positively correlated with BUN values at baseline (r=0.54667, p=0.0154). After RHDV infection, the serum concentrations of CREA, Na\(^+\), and K\(^+\) were significantly and positively correlated with BUN and Cl\(^-\) negatively (Table 2). The changes of CREA correlated with BUN better as indicated with higher correlation coefficient and R-squared. During this acute infection of RHDV, change in serum Na\(^+\) correlated with all the other parameters. The K\(^+\) was correlated with CREA better than with
BUN and CREA are preliminary indices of glomerular filtration. Consistent elevation of BUN levels occurs only when the renal function, specifically the glomerular filtration rate (GFR), is reduced by 40 to 60 percent [22]. In this study, renal function impairment was demonstrated by the elevation of BUN and CREA. As with BUN, the reduced GFR increases the serum concentration of CREA [13], which explained the strongest correlation of BUN with CREA in correlation analysis (Table 1). The increases in BUN and CREA concentrations conform to the study of Ferreira [7]. The significant elevation of BUN and Osmolarity were presented at 36 HPI with the appearance of DIC in kidney specimens. The increase in BUN levels was 6 hr earlier than CREA and might be due to the increase in catabolism caused by fever and infection, because CREA is not usually significantly affected by catabolic factors [11]. The significant increases in BUN and CREA were later (at 36 HPI and 42 HPI) than the increase of ALT and AST in our previous study (at 24HPI and 18HPI), which resembled the finding of others in which RHDV RNA presents as early as...
of differences in tubular re-absorption and diffusion rates, due to the effects of diet and protein metabolism on the two compounds [11]. The increase of BUN/CREA ratio revealed that the glomerular filtration function was damaged more severely than the re-absorption function. The elevations in BUN, CREA and BUN/CREA ratio fulfill the criteria of the developing of pre-renal acute renal failure [4]. The impairment of secretory function of kidney facilitated the development of hyperkalemia, which was supported by the results that the change of K⁺ was more strongly correlated with BUN and CREA than Na⁺ (Table 2). Following the disease progression, the number of rabbits with CREA over 3 mg/dl increased from 24 HPI (6%, n=17) through 42HPI (67%, n=6) (Fig. 3B), along with hyperkalemia development and DIC found in pathologic examination. All these three changes suggested the development of tubular injury. This finding is consistent with the apoptosis of tubular cells in kidney at late post-infection period [2].

The Na⁺ concentration determines the osmotic pressure of extracellular fluid (ECF), and K⁺ determines the intracellular fluid (ICF) osmolality. Poor water intake after infection might induce dehydration heading to elevate serum Na⁺ in the rabbits. The loss of cell membrane integrity (as massive hepatic necrosis in RHD here) produces efflux of K⁺ from ICF to the ECF. Normally, most of the excess K⁺ is excreted in urine (kaliuresis) [11] when renal function was efficient. In this study, serum K⁺ decreased at 18 HPI and 24 HPI first when the renal function was still effective. However, following the decrease in renal function (BUN significantly elevated at 36 HPI), excess K⁺ would be retained instead of excreted in the urine. Additionally, the induced severe hepatic necrosis might release of a large amount of K⁺ resulting in hyperkalemia. Hyperkalemia can lead to life-threatening cardiac conduction abnormality that is bradycardia [11] and also resulted in DIC.

Osmolality is the number of solute particles per unit weight of solution and about equal to osmolarity. For most calculations, the Osmolal gap, numerical difference between osmolality, ranges from –5 to 15 mOsm/kg [11]. In this study, the significant increase in three effective osmoles, Na⁺, K⁺ and BUN, produced significant hyperosmolality at 36 HPI (Fig. 2D) with Osmolal gap values > 20 mOsm/kg. Consequently, the internal balance disturbed, fluid balance might change and cause interference in the diffusion of metabolites leading to systemic impairments in organs.

The baseline value was obtained after 8 hr of fasting. However, to imitate the field situation, the food and water wasn’t deprived during experiment. Hence, the slightly increase in glucose level at 18 HPI (Fig. 2E) was probably a result from food supplement after virus inoculation. Death of sick rabbit was partly proceeding by hypoglycemia. Hypoglycemia significantly developed from 24 HPI which was 12 hr earlier than that reported by Tunon [23]. Loss of appetite might not be the major reason, because even after 96 hr of fasting, no significant change was evident [24]. This occurs, because there was a bolus of food that is being
continuously digested in the intestine throughout this fasted period [24]. In view of the big individual variation, the mean of serum glucose concentration was relatively normal. Even at 30 HPI, the lowest mean values (51.72 ± 21.74 mg/dl) was still over 50 mg/dl. After calculating the frequency distribution, it was suggested that all rabbits that died (Table 1) had values below 50 mg/ml before dying. Marked depletion of liver glycogen seen by transmission electron microscopy was found in late stage of RHD infection [6], representing destruction of small endoplasmic reticulum system and resulted in the impairment of the detoxification ability of liver. In addition to the destruction of hepatocytes

![Fig. 4. Changes of the microscopic findings in liver specimens at different hr post inoculation. (A) Degeneration and necrosis of hepatocytes and congestion at portal area. Died at 32 HPI. H&E stain. Bar=50 µm. (B) Fatty change at portal area. Died at 37 HPI. H&E stain. Bar=50 µm. (C) Necrosis close to central vein. Died at 45 HPI. H&E stain. Bar=50 µm. (D) Mononuclear cell infiltration at portal area. Be sacrificed at 66 HPI. H&E stain. Bar=50 µm.](image)

<table>
<thead>
<tr>
<th>Table 1. The concentrations of serum parameters in RHD rabbits prior to death</th>
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<tr>
<td>Parameter</td>
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<tr>
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<tr>
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<tr>
<td>Died (n=10):</td>
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<tr>
<td>&gt;NR (%)*:</td>
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<tr>
<td>Sacrificed (n=3):</td>
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<tr>
<td>&gt;NR (%)*:</td>
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<td>Significance:*</td>
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* Frequency of rabbits with individual value higher than normal range (NR) of baseline value.
for insufficient functional cells to carry out efficient glycogen storage and gluconeogenesis [4], loss of appetite also contributed to the severe hypoglycemia. Since the brain relies on glucose as its primary energy source, hypoglycemia thus elicits symptoms related to altered cerebral function. Therefore, coma developed clinically followed by death.

The RHDV infection reproduces clinical, biochemical, and histological features of the fulminant liver failure syndrome and satisfies criteria for suitable animal model [23]. Study on the vaccination and treatment of RHD might contribute greatly in human viral hepatitis therapy. Nephropathy is also associated with human hepatitis virus [3, 18]. In the present study, RHDV infection resulted in renal dysfunction following the progression of liver injury (Figs. 4A-D). It is thus suggested that BUN and CREA are proper parameters for rabbit renal function monitoring after RHDV infection. Although renal insufficiency, as suggested by the increases in BUN and CREA, did not appear to contribute to the coma or death in rabbits without hyperkalemia or hypoglycemia, the consequent significant change in electrolytes resulted in the increase of ECF osmolality and flow disturbance that destroyed homeostasis in cells might play an important role in causing the death. Fulminant liver disease is regarded as a group of systemic diseases with the main focus of illness in the liver, rather than a specific disease of a single organ, the liver [16]. Therefore, the later impairments in renal function and electrolyte balance might be an important threat for rabbits which survived from acute fulminant hepatitis in RHD, and the measurements of renal function and electrolytes as well as liver functional parameters for monitoring the progression in a fulminant viral hepatitis as an index for treatment are necessary.

REFERENCES


### Table 2. Correlation analysis among serum parameters in RHDV infected New Zealand White rabbits in 66 hr

<table>
<thead>
<tr>
<th></th>
<th>CREA</th>
<th>Na</th>
<th>K</th>
<th>Cl</th>
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<tbody>
<tr>
<td>BUN</td>
<td>R²</td>
<td>0.8078</td>
<td>0.0332</td>
<td>0.1021</td>
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<tr>
<td>r</td>
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<td>0.4592</td>
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<tr>
<td>p</td>
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<table>
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<tr>
<th></th>
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<th>Na</th>
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<tbody>
<tr>
<td></td>
<td>R²</td>
<td>0.0133</td>
<td>0.1038</td>
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<tr>
<td>p</td>
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R²: R-squared, r= Pearson correlation coefficients, p= Probability.


