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Research Article

Hyperproteinemia in Rabbit Hemorrhagic Disease

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ABSTRACT

Rabbit hemorrhagic disease virus (RHDV) induced fulminant hepatitis in adult rabbits which were similar to those in human fulminant. Detail serum biochemistry in New Zealand White rabbits infected with rabbit hemorrhagic disease was studied in order to establish initial screening criteria for the disease and identification of affected animals for further medical studies. Rabbits were each inoculated with one 50% rabbit lethal dose (RLD_{50}) of rabbit hemorrhagic disease virus. Blood samples were collected at 0 hours post inoculation (HPI), 18 HPI and every 6 hours thereafter. After RHDV inoculation, highly significant increases of alanine aminotransferase (ALT), alkaline phosphatase (ALP) and albumin (ALB) ($p<0.0001$) were noted; as well as significant increases ($p<0.05$) in total protein (TP) and globulin (GLO). The female had higher ALB ($p=0.028$) and A/G ($p=0.0037$) values than the sample.

Keywords: *liver, rabbit hemorrhagic disease virus, serum protein*

INTRODUCTION

Rabbit hemorrhagic disease (RHD) was first reported in 1984 in China (Liu et al., 1984) and disperses into Europe, the Middle East and Asia. This lagomorph disease is a disease mandated to be reported to World Organization for Animal Health, and considered to be of socio-economic importance and significant in the international trade of animals and animal products. RHD also severely damaged the rabbit-raising industry and related bio-product industries in Taiwan. The virus had been well characterized by hemagglutination test, electron microscopic examination, viral protein analysis and RT-PCR in 1998 (Shien et al., 1998). Importation of domestic rabbits or contaminated rabbit foods was presumed to be responsible for the introduction of RHD into Taiwan.

RHDV caused as an acute fatal necrotic viral hepatitis in both wild and domestic adult rabbits (Cooke et al., 2000). In laboratory experiments, up to 85% adult (older than 2 months) rabbits died within 36 to 72 hours after infection (Marcato et al., 1991; Tunon et al., 2003), whereas younger rabbits usually survive and become RHD virus (RHDV) carriers (Xu et al., 1989). The highly contagious agent which belongs to Caliciviridae is a single strand, non-enveloped, RNA virus (Ohlinger et al., 1990). The viral particles were mainly accumulated in the membrane-bound cisternae and scattered around the vacuoles in the cytoplasm of many necrotic hepatocytes (Park et al., 1992). Clinicopathologic parameters used to evaluate the liver can be divided into serum enzyme tests and function tests. The serum biochemistry tests such as AST, ALT, ALP, total bilirubin, direct bilirubin, and albumin were recommended for diagnosis and monitoring of hepatic injury (Dufour et al., 2000). In this study we measured serum ALT, ALP, total bilirubin (TB), total protein (TP), albumin (Alb) and globulin (Glo) to understand the impact of RHD in liver enzymatic and functional systems.

MATERIALS AND METHODS

Nineteen, 15-week-old, clinically healthy RHD-free

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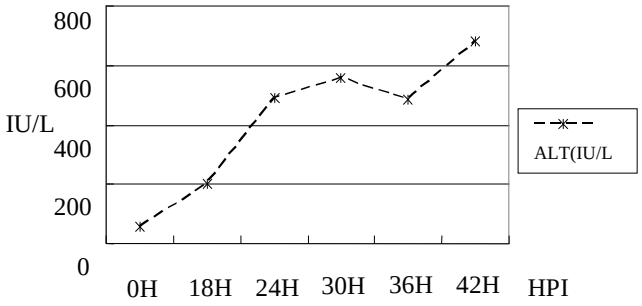

Fig. 1 Changes in ALT in 42 hours after RHDV infection

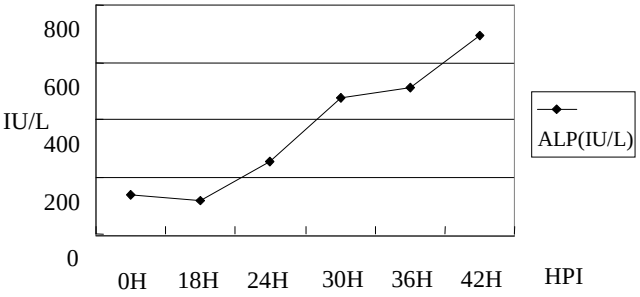

Fig. 2 Change in ALP(IU/L) in 42 hours after RHDV infection

New Zealand White rabbits, as confirmed by the absence of antibody against RHDV in the hemagglutination inhibition (HI) test, were obtained from the National Animal Health Research Institute in Taiwan. The micro-organism monitoring of the rabbits in the institute includes the following diseases: rabbit hemorrhagic disease, myxomatosis, tularemia, pasteurellosis, bordetellosis, salmonellosis, pseudomoniasis, coccidiosis, aural acariasis, and oxyurisosis (pinworm). None of the rabbits, had previously been vaccinated with inactivated RHD vaccine. The rabbits were kept in sanitized wire cages and separated by sex. All were fed commercial dry rabbit feed that contained 19% crude protein, 10% crude fiber and 2,500 kcal/kg metabolic energy ("Laboratory Rabbit" feed, Fwu-Sow Industry Co., Taiwan). The rabbits were kept and handled according to the regulations laid down by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University.

The RHDV was obtained from a liver specimen of a field case. A 10 % suspension of homogenized liver in 0.01 M phosphate-buffered saline was made, and centrifuged at 7000 x g for 30 minutes at 4℃. The supernatant was then filtered through a 0.22 μm filter and stored at -70℃ until use. A 50% rabbit lethal dose (RLD50) viral titration of the supernatant was carried out by intra-muscularly inoculating groups of four 2-month-old rabbits (weighing over 2 kg) with serial dilutions of 10^{-4} to 10^{-9} in Eagle's minimal essential medium. A total of 24 rabbits, 4 rabbits for each titer, were used. The rabbits were monitored continuously for 7 days. Rabbits that died with typical RHD signs of hemorrhages in the respiratory system, liver, spleen and cardiac muscle were recorded. The Reed and Muench method was used to calculate the RLD_{50} (Davis et al., 1990).

In the present experiment, 19 rabbits were each inoculated intramuscularly with one RLD50 RHDV. Blood samples were collected from the auricular artery of the rabbits after 8 hours of fasting and used as a base line (0 hour post-inoculation; HPI). After virus inoculation, 1.5 ml blood samples were collected at 18 HPI, and then every 6 hours until a rabbit died or until 42 HPI. Blood collected in syringes without anticoagulant was left at room temperature for 30 minutes and then at 4℃ for 30 minutes to elute the serum. Whole blood was then centrifuged at 1000 x g for 10 minutes and the sera co -llected.

The ALT, ALP, TP, ALB, GLO, T-BIL, and A/G ratio were measured by an autoanalyzer (7150, Hitachi Co., Japan) using spectrophotometic method. Data collected at different HPI were analyzed using Statistic Analysis System (SAS 9.1). The means and standard deviations were calculated by the Means Procedure for time and sex. Least Squares Means of the General Linear Model (GLM) Procedure were applied to the analysis of the differences on the effect of time and sex. P values less than 0.05 were considered significant. The strength of the relationship that existed between the parameters was defined by the statistics of the Pearson correlation coefficient and its significance by the correlation procedure. Regression analysis was used to determine the increasing pattern.

RESULTS

Changes in ALT and ALP

Serum ALT markedly elevated highly significantly from 18 HPI (Fig. 1) without sex difference. The values were peaked at 42 HPI with maximal increment of 11 times higher than mean at 0 HPI for ALT. The timing of 100% rabbits with all values over the normal values were 30 HPI for ALT. There was no significant difference of rabbits which died or survived.

Significant increase in serum ALP was observed later at 30 HPI (Fig. 2). The mean values also peaked at 42 HPI with increments of about 5 folds higher of the 0 HPI mean for ALP. No sexual difference was observed.

Changes in TP, ALB, GLO, A/G ratio and T-BIL

Time differences were also significant among TP,

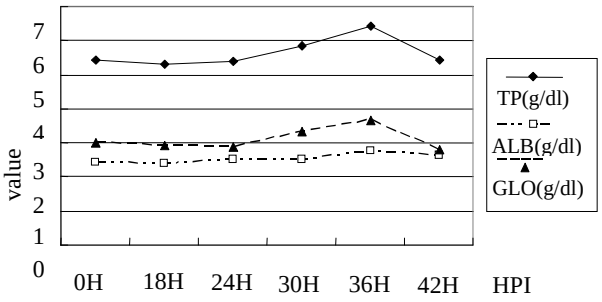

Fig. 3A Change in TP, ALB and GLO in 42 hours after infection

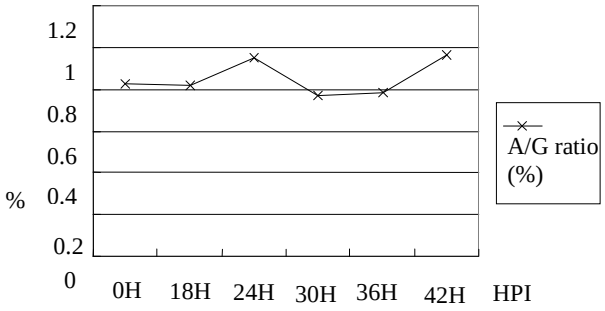

Fig. 3B Change in A/G ratio in 42 hours after RHDV infection

GLO and ALB (Fig. 3A) but not in A/G (Fig. 3B) and T-BIL (Fig. 4). The TP and GLO changed in parallel. Though changed in similar trend with TP and GLO, the ALB concentration was over the baseline values from24HPI through 42HPI. The female had higher mean values than the male did (ALB: 2.55±0.18 g/dl vs. 2.44±0.19 g/dl, p=0.0111; A/G: 0.9 ±0.26% vs. 0.79±0.11%, p=0.028). TheTP values positively correlated with GLO higher than ALB with higher r and R-square values (Table 1). Changes in the ALB/TP and GLO/TP ratios both weren't achieved significant level (p=0.09). T-BIL was not affected by either time or sex effects (Fig. 4).

Regression analysis

By regression analysis, parameters measured above can be divided into two groups. The ALT values versus time fit logarithmic curve significantly better, and the quadratic regression line fit ALP, TP, and ALB better.

Correlation Coefficient analysis among parameters

Among all parameters measured in this study, only ALB significantly and positively correlated with ALT before inoculation (r=0.5557, p=0.0285). While after RHDV infection, the ALP, TP, ALB, and T-BIL were significantly and positively correlated with ALT (Table 1). Change of T-BIL significantly correlated with ALT positively (Table 1) , but insignificantly correlated with ALP. The ALB values significantly correlated with ALP better than ALT with higher R-Squares and Pearson

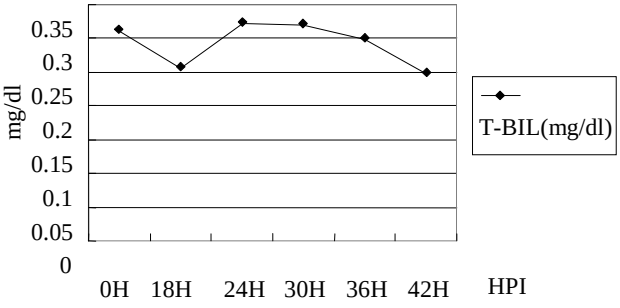

Fig. 4 Change in T-BIL in 42 hours after RHV infection

Correlation Coefficients. Serum GLO and A/G were neither correlated with ALT nor with ALP. The TP significantly correlated with GLO (Table 1).

DISCUSSION

ALT is the most specific and routinely measured enzyme in diagnosis of hepatic function in the dogs, cats and primates (Meyer et al., 1992; Bush, 1991). In this study, the ALT elevated first at 18 HPI and the logarithmic expansion in ALT meant the damage of heaptocytes were quickly. A rise in ALP activity is the most sensitive indicator of cholestasis because it develops before there is any detectable increase in plasma bilirubin (Bush, 1991). This could explain the change in ALP was significant while change in T-BIL was insignificant. Besides, change of T-BIL significantly correlated with ALT but did not correlated well with ALP, which indicated that the increase of T-BIL (Fig. 6) was mainly resulted from hepatocyte injury instead of the obstruction of bile flow at early stage in RHD.

High enzyme activities reflect the number of cells involved but not necessarily suggestive of a loss of organ function, not until a high proportion (e.g. 75%) of the liver cells were severely affected (Bush, 1991). For understanding of the impairment of liver function by RHDV infection, we measured concentrations of protein levels including TP, ALB, GLO, A/G, and T-BIL. Since albumin and all other proteins, except for the immuneglobulins, are synthesized by the liver (Bush, 1991; Latimer et al., 2003), when liver injury occurred, protein synthesis would be impaired and resulted in hypoalbuminemia. Furthermore, proteins are synthesized on ribosomes of the rough endoplasmic reticulum and transported in small membrane-bound vesicles (Burkitt et al., 1997) , so they are not easily released, the plasma half-life of albumin is typically 19-21 days in human (Dufour et al., 2000), 8 days in dog and 19 days in horse (Latimer et al., 2003). Therefore, plasma albumin seldom is decreased in acute hepatitis because of its long half-life (Dufour et al., 2000). All these explained why the values of ALB were not decreased in this study. Acute inflammation (e.g. acute hepatitis) and fever usually produce an increase in

Table 1. Correlation ananlysis among serum parameters in RHDV infected New Zealand White rabbits in 42 hours post inoculation

		ALP	TP	ALB	T-BIL
ALT	R^2	0.2739	0.1080	0.1387	0.1833
	r	0.5224	0.2801	0.2690	0.4486
	p	<.0001	0.0383	0.0471	0.0006
		ALT	ALB		
ALP	R^2	0.2739	0.3239		
	r	0.5224	0.5710		
	p	<.0001	<.0001		
		ALB	GLO	A/G	
TP	R^2	0.2434	0.9073	0.4272	
	r	0.4577	0.9551	-0.6057	
	p	0.0002	<.0001	<.0001	
		A/G	AFP		
ALB	R^2	0.0825	0.1012		
	r	0.3520	0.3084		
	p	0.0050	0.0147		
		A/G			
GLO	R^2	0.6212			
	r	-0.7882			
	p	<.0001			

R2: R-squared p: probability

r: pearson correlation coefficients

α-globulin (acute phase reactants) (Bush, 1991; Latimer et al., 2003), there was a high correlation coefficient of TP vs GLO (R^2=0.9122). According to our previous study (data not shown), the normal female had significantly higher ALB values. Therefore, the significant difference of ALB in genders after RHDV infection might be due to physical difference in sex instead of virus affinity. The stronger correlation between ALB and ALP than with ALT revealed that change of ALB was later and induced coordinately by severer damage in hepatocytes following the massive leakage of cytoplasmic enzymes. This is the first demonstration that fulminant RHDV infection leads to hyperproteinemia.

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