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Adverse fetal outcomes in pregnant rabbits experimentally infected with rabbit hepatitis E virus

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ABSTRACT

Hepatitis E virus (HEV) causes severe hepatitis in pregnant women, with associated poor fetal outcomes. To study HEV viral pathogenesis, pregnant rabbits were infected with low- and high-dose rabbit HEV at 2 weeks gestation. HEV was identified in the serum, feces, and liver tissue of infected rabbits, and dose-dependent fetal mortality rates ranging from 67% to 80% were observed. The aspartate transaminase (AST)/alanine transaminase ratio was significantly higher ($P < 0.01$) in high-dose infected rabbits than low-dose infected and negative control rabbits 14 days post infection (dpi). Tumor necrosis factor-α (TNF-α) was significantly higher in low-dose ($P < 0.01$) and high-dose infected rabbits ($P < 0.001$) than in negative controls 7 dpi. High-dose HEV-infected rabbits produced significantly more interferon-γ (IFN-γ; $P < 0.05$) than negative control rabbits at 7 and 14 dpi. High levels of AST, TNF-α, and IFN-γ may substantially influence adverse fetal outcomes in pregnant rabbits infected with high-dose HEV.

1. Introduction

Hepatitis E virus (HEV) belongs to the family *Hepeviridae*, which contains two genera, *Orthohepevirus* and *Piscihepevirus*. The genus *Orthohepevirus* includes four species; *Orthohepevirus* A to D, and *Orthohepevirus A* is further divided into seven genotypes, HEV-1 to HEV-7 (Smith et al., 2014). HEV-1 and HEV-2 are endemic in developing countries, especially in Asia and Africa, and only infect humans (Huang et al., 1992; Tam et al., 1991). The drinking of contaminated water is the main cause of large outbreaks of hepatitis in those regions (Kumar et al., 2013). HEV-3 and HEV-4 are found worldwide, and infect both humans and animals, including pigs and wild boars (Caruso et al., 2015a; Meng et al., 1998, 1997; Wang et al., 2000). Most human infections with these viruses are caused by consumption of raw or undercooked meat, and they are therefore considered zoonotic pathogens (Kumar et al., 2013; Meng, 2011). HEV infection induces self-limiting acute hepatitis in most cases, with a mortality rate of approximately 2% in the general population. However, HEV infection in pregnant women on the Indian subcontinent and elsewhere results in severe complications, with 20–30% maternal mortality rates and adverse fetal outcomes including abortion, stillbirth, and intrauterine death (Kumar et al., 2013; Shalimar and Acharya, 2013; Shinde et al., 2014). Fulminant hepatic failure is commonly observed in pregnant women (Patra et al., 2007; Shalimar and Acharya, 2013). Unfortunately, the molecular mechanisms underlying HEV infection in pregnant women remain to be fully elucidated.

HEV has been isolated from farmed rabbits in several countries, including China (Cossaboom et al., 2011; Izopet et al., 2012; Zhao et al., 2009), and has also been found in wild and pet rabbits (Burt et al., 2016; Caruso et al., 2015b). Rabbit HEV (rHEV) is genetically similar to zoonotic HEV-3 strains, but is genetically distinct from other HEV genotypes (Caruso et al., 2015b; Cossaboom et al., 2011). Viral infectivity and virulence has been demonstrated in rabbits through experimental inoculation. Viremia, fecal viral shedding, and hepatitis are observed in infected rabbits (Ma et al., 2010). Non-human primates experimentally infected with rHEV demonstrate viral replication and acute hepatitis, similar to rabbits (Liu et al., 2013). The virus also infects pigs; however, its infectivity and replication level are relatively low (Cossaboom et al., 2012). A recent study demonstrated that pregnant rabbits infected with rHEV experience infertility, miscarriage, and high maternal mortality after delivery (Xia et al., 2015). However, the study provided limited explanation of the molecular mechanisms

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underlying these adverse effects. Additional research is therefore required to understand the pathogenic mechanism by which rHEV affects maternal and fetal outcomes in pregnant rabbits.

In the present study, pregnant rabbits were infected with low or high dose of a rHEV strain isolated in Korea. Adverse fetal outcomes were observed in the rHEV-infected rabbits in a dose-dependent manner. We also present evidence of the molecular mechanisms by which rHEV causes its effects.

2. Materials and methods

2.1. Pregnant rabbits and progesterone measurements

Seven-month-old, pregnant, specific pathogen-free rabbits, with body weights ranging from 5.0 to 5.5 kg, were obtained from OrientBio (Seongnam, Korea). Their pregnancy status was determined by measuring the progesterone levels in their serum samples by electrochemiluminescence immunoassay at Neodin Veterinary Laboratory (Seoul, Korea). The HEV-free status of the rabbits was confirmed by testing serum samples with a commercial HEV enzyme-linked immunosorbent assay (ELISA) kit (Wantai, Beijing, China). Horseradish peroxidase-labeled goat anti-rabbit IgM mu chain (Abcam, Cambridge, UK) and goat anti-rabbit IgG (Abcam) were used as the conjugates for detection of rabbit IgM and IgG, respectively. The HEV-free status of the rabbits was also determined by testing fecal samples with a nested reverse transcription-polymerase chain reaction (RT-PCR) specific to rHEV before inoculation. The sensitivity and specificity of the nested RT-PCR was confirmed in a previous study (Ahn et al., 2017).

2.2. Virus

The KOR-Rb-1 HEV strain, belonging to genotype 3 (GenBank accession number KY496200), was obtained from rabbit fecal samples and used to inoculate the pregnant rabbits (Ahn et al., 2017). Briefly, 1 g of fecal sample was suspended in phosphate-buffered saline (PBS; pH 7.4) and centrifuged at 3000 × *g* for 20 min. The supernatant was collected, filtered through a 0.2 μm syringe filter, and stored at −70 °C until use. The genome equivalents (GE) of rHEV were determined by RT-PCR as previously described (Jothikumar et al., 2006). The virus was adjusted to 10^3 (low dose) and 10^6 (high dose) GE/mL from fecal supernatant initially containing $10^{6.2}$ GE/mL, and used to inoculate pregnant rabbits.

2.3. Experimental design and sample collection

All animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Konkuk University, Korea (permit number KU16128). A total of 18 pregnant rabbits were divided equally into three groups: negative control, low-dose infection, and high-dose infection. Rabbits in the negative control group were intravenously inoculated with 1 mL of PBS. Rabbits in the low- and high-dose infection groups were intravenously inoculated at two weeks gestation with 10^3 and 10^6 GE of rHEV, respectively. Serum and fecal samples were collected before inoculation, and again at 7 and 14 days post inoculation (dpi). Supernatants were obtained from fecal samples as described above. The fecal supernatants and serum samples were stored at −70 °C. All rabbits were euthanized within two days of delivery of offspring (16 dpi), and their liver tissues were stored in formalin.

2.4. Detection of rHEV RNA by RT-PCR

HEV RNA was extracted from fecal and serum samples using a Patho Gene-spin DNA/RNA Extraction Kit (Intron, Korea) according to the manufacturer's instructions, and stored at −70 °C until use. HEV was detected by nested RT-PCR according to Ahn et al. (2017). PCR products were identified by DNA sequencing.

2.5. Detection of alanine transaminase (ALT), aspartate transaminase (AST), and HEV antibodies

ALT and AST concentrations in serum samples were determined at Neodin Veterinary Science Institute (Seoul, Korea) by UV-assay, according to the International Federation of Clinical Chemistry and Laboratory Medicine, without pyridoxal phosphate activation. Baseline ALT and AST levels were determined using pre-inoculation samples. The serum samples were also tested for IgM and IgG against rHEV. Antibody titers were measured using a commercial ELISA kit as described in a previous section (Wantai, Beijing, China). Sample/cutoff values were calculated, and values > 1 were considered positive.

2.6. Measurement of cytokine levels

The levels of interleukin (IL)-2, IL-4, IL-10, IL-12, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) in pregnant rabbit serum samples were determined using commercial ELISA kits, according to the manufacturers' instructions: rabbit IL-2 DuoSet ELISA kit (R & D Systems, Minneapolis, MN, USA), rabbit IL-4 DuoSet ELISA kit (R & D Systems), rabbit IL-10 ELISA kit (Cusabio, Wuhan, China), rabbit IL-12 ELISA kit (FineTest, Wuhan, China), rabbit TNF-α ELISA kit (Cusabio), and rabbit IFN-γ ELISA kit (Raybio, Norcross, GA, USA). All serum samples were tested in duplicate.

2.7. Histopathology

Liver tissues collected during necropsy were fixed overnight with 10% neutral buffered formalin and embedded in paraffin according to standard tissue processing protocols. For histopathological examination, each slide was cut to 4 μm thickness and stained with hematoxylin and eosin (H & E). Two veterinary pathologists evaluated the histopathology. Immunohistochemistry was performed using the standard avidin/biotin complex (ABC) method. Briefly, unstained slides were deparaffinated and rehydrated. Antigen retrieval was performed with 20 μg/mL proteinase K (Gibco BRL, Carlsbad, CA, USA) at 37 °C for 20 min. Samples were blocked with 10% normal horse serum (Vector Laboratories, Burlingame, CA, USA) in PBS (0.01 M, pH 7.4) for 30 min at room temperature. Rabbit polyclonal anti-HEV antibody (Biorybt, San Francisco, CA, USA) was applied to the slides at a dilution of 1:50 in antibody diluent (DAKO, Carpinteria, CA, USA) and incubated at 4 °C overnight. Biotinylated horse anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. ABC-alkaline phosphatase (Vector Laboratories) was applied as a substrate. Visualization was performed by immersing the sections in a solution of Vector Red® substrate (Vector Laboratories) for 5 min at room temperature. Mayer's hematoxylin was used as a counterstain.

2.8. Statistical analysis

The data were analyzed using two-way analysis of variance in GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA). A Bonferroni multiple comparisons test was performed as post hoc analysis. Differences were considered significant at P-values < 0.05.

3. Results

3.1. Adverse fetal outcomes in pregnant rabbits infected with rHEV

The pregnancy status of the rabbits was determined by measuring the serum progesterone concentration until delivery (Supplementary Fig. 1). The serum progesterone concentrations in all except one rabbit (#15) were between 6.0–22.8 ng/mL at 2 weeks gestation, and

Table 1
Pregnancy outcomes in pregnant rabbits infected with HEV.

Group	ID[a] of pregnant rabbit	Number of miscarriages	Number of stillbirths	Number of normal offspring	Mean number of normal offspring	Fetal mortality rate
Negative control	1	0	0	6	8.16	0%
	2	0	0	11		
	3	0	0	10		
	4	0	0	8		
	5	0	0	7		
	6	0	0	7		
Low-dose HEV[†]	7	0	1	9	5.6	67%
	8	0	0	8		
	9	0	2	4		
	10	0	3	0		
	11	0	2	4		
	12	0	0	9		
High-dose HEV[‡]	13	1	0	0	3.4	80%
	14	0	1	0		
	15	NA[b]	NA	NA		
	16	0	4	4		
	17	0	0	11		
	18	1	1	2		

[a] ID: identification number.
[b] NA (not available): Rabbit #15 was not pregnant.
[†] Infection with 10^3 GE of rabbit HEV.
[‡] Infection with 10^6 GE of rabbit HEV.

gradually decreased to the negligible range (0.5–1.4 ng/mL) at birth (Supplementary Fig. 1A to C). The progesterone concentration in rabbit #15 was < 1.0 ng/mL throughout the experimental period, indicating that it was not pregnant (Supplementary Fig. 1C), and it was therefore excluded from the study. The remaining 5 rabbits in the high-dose HEV infection group were compared with the negative control and low-dose HEV infection groups. Rabbits in the negative control group delivered between 6 and 11 normal offspring, with no miscarriages or stillbirths (Table 1). However, most rabbits receiving low- and high-dose HEV delivered dramatically fewer live offspring. Four rabbits in the low-dose group (#7, #9, #10, and #11) and three in the high-dose group (#14, #16, and #18) had at least one stillborn offspring. Miscarriage was observed in two rabbits (#13 and #18) infected with high-dose HEV. However, intrauterine fetal death could not be demonstrated, as no non-viable fetuses were identified in the uteruses of rabbits from any group during autopsies at the end of the experiment. Two rabbits in the low-dose HEV group (#8 and #12) and one in the high-dose group (#17) produced normal offspring without any observed miscarriages or stillbirths. The average number of normal offspring delivered by rabbits in the negative control, low-dose, and high-dose HEV infection groups were 8.16, 5.6, and 3.4, respectively. The overall fetal mortality rates of rabbits in the negative, low-dose, and high-dose HEV infection groups were 0%, 67%, and 80%, respectively. These data demonstrate a dose-dependent increase in the rate of fetal mortality by pregnant rabbits infected with rHEV. We investigated the possibility of HEV transmission to fetuses and offspring by nested RT-PCR, and the virus was not detected in any miscarried fetuses, stillborn offspring, or normal offspring immediately after birth (data not shown).

3.2. Replication of rHEV in pregnant rabbits

The active replication of rHEV in pregnant rabbits was measured using serum and fecal samples collected from the three groups. As expected, HEV was not detected in rabbits in the negative control group during the experimental period (Table 2). However, intermittent viremia and fecal viral shedding were observed in serum and fecal samples obtained from rabbits infected with HEV, between 7 and 14 dpi. At 14 dpi, serum viremia was identified in 50% and 40% of rabbits infected with low-dose and high-dose HEV, respectively, and viral shedding was observed in 50% and 80% of rabbits infected with low-dose and high-dose HEV, respectively. Therefore, both doses of rHEV caused active infection in pregnant rabbits.

3.3. Antibody production in pregnant rabbits infected with rHEV

Production of IgM and IgG antibodies against rHEV was measured in serum samples collected from pregnant rabbits 0, 7, and 14 dpi. IgM antibodies were not detected in any serum samples (data not shown). IgG antibodies were detected in only one rabbit (#14) in the high-dose infection group (data not shown).

3.4. Acute hepatitis in pregnant rabbits infected with rHEV

The levels of the liver enzymes ALT and AST were measured in serum samples. Normal ALT and AST ranges were observed in rabbits in the negative control, low-dose, and high-dose infection groups 0 dpi, as expected (Fig. 1A and B). The ALT and AST levels remained in the normal range 7 dpi in all three experimental groups. However, at 14 dpi the ALT and AST levels were significantly higher in rabbits infected with high-dose HEV than in negative control rabbits ($P < 0.001$ and $P < 0.05$ for ALT and AST, respectively). In contrast, at 14 dpi the ALT and AST levels of rabbits infected with low-dose HEV remained in the normal range, with no significant increase compared to the negative controls. The AST/ALT ratio was significantly higher ($P < 0.01$) 14 dpi in rabbits infected with high-dose HEV (Fig. 1C). Interestingly, rabbit #17 in the high-dose infection group, which delivered normal offspring, displayed no increase in either enzyme level (data not shown).

3.5. Cytokine levels in pregnant rabbits infected with rHEV

The levels of TNF-α, IL-2, IL-4, IL-10, IL-12, and IFN-γ were determined in serum samples. The amount of TNF-α in rabbits infected with low- and high-dose HEV was significantly higher at 7 dpi ($P < 0.01$ and $P < 0.001$, respectively), than the negative control rabbits (Fig. 2A). At 14 dpi, TNF-α levels in rabbits infected with high-dose HEV were significantly higher ($P < 0.05$) than in rabbits in the negative control and low-dose HEV infection groups. The amount of IFN-γ in high-dose HEV rabbits was significantly greater ($P < 0.05$) than in rabbits in the negative control group 7 and 14 dpi (Fig. 2B). The other cytokines did not significantly increase 7 dpi in any rabbits. However,

Table 2
Detection of HEV RNA in serum and fecal samples collected from rabbits infected with HEV.

	Negative control		Low-dose HEV		High-dose HEV	
	Serum (%)	Feces (%)	Serum (%)	Feces (%)	Serum (%)	Feces (%)
0 dpi[a]	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	0/5 (0)	0/5 (0)
7 dpi	0/6 (0)	0/6 (0)	1/6 (17)	2/6 (33)	1/5 (20)	2/5 (40)
14 dpi	0/6 (0)	0/6 (0)	3/6 (50)	3/6 (50)	2/5 (40)	4/5 (80)

[a] dpi: days post infection.

14 dpi, the levels of the Th1-type cytokines IL-2 and IL-12 were significantly higher in the high-dose HEV rabbits than in the negative control and low-dose HEV rabbits ($P < 0.001$ and $P < 0.05$ for IL-2 and IL-12, respectively; Fig. 2C and D). In addition, significantly higher amounts of the Th2-type cytokines IL-4 and IL-10 were identified in high-dose HEV rabbits compared to those in the negative control and low-dose HEV groups at 14 dpi ($P < 0.05$ and $P < 0.01$ for IL-4 and IL-10, respectively; Fig. 2E and F). The IL-12/IL-10 ratios of rabbits in each group were similar at 14 dpi (data not shown).

3.6. Histopathology

Rabbits in the negative control group had no inflammatory lesions or HEV antigens in their liver tissues (Fig. 3A and B). However, mild to moderate lymphocytic hepatitis was observed in the liver tissues of rabbits infected with low- and high-dose rHEV (Fig. 3C and E). By immunohistochemistry, rHEV antigens were identified in the cytoplasm of hepatocytes near hepatic central veins (Fig. 3D and F). The intensity of inflammation and immunohistochemical signals increased in a dose-dependent manner in HEV-infected rabbits.

4. Discussion

Pregnant women are prone to infection with HEV, and develop more severe hepatitis than non-pregnant women and male adults (Khuroo et al., 1981; Shalimar and Acharya, 2013). HEV infection during pregnancy has been reported to induce high rates of maternal mortality and lead to poor fetal outcomes in women living in specific regions, such as the Indian subcontinent (Kumar et al., 2004; Patra et al., 2007; Shinde et al., 2014). However, other studies have found no evidence of increased mortality in pregnant women infected with HEV (Bhatia et al., 2008). Similarly, worse fetal and maternal outcomes could not be identified in pregnant pigs and rhesus monkeys experimentally infected with HEV (Kasorndorkbua et al., 2003; Tsarev et al., 1995). The reason for these contradictory observations is still poorly understood.

In the present study, pregnant rabbits infected with rHEV were used as an animal model to explore the adverse fetal or maternal outcomes observed in many HEV-infected pregnant women. High rates of fetal mortality (67–80%) were observed in pregnant rabbits infected with HEV. This is consistent with a recent study on pregnant women infected with HEV in India, where 71% of cases had adverse fetal outcomes and 32% resulted in maternal death (Shinde et al., 2014). Another study observed a miscarriage rate of 33% and a maternal death rate of 50% in pregnant rabbits infected with rHEV isolated in China (Xia et al., 2015). In that study, maternal fatality was observed 6–7 weeks after delivery. However, the rabbits in our experiments were euthanized within two days after delivery of offspring and no maternal death was observed during the experimental period. These differences could be attributed to experimental conditions and the virulence of the rHEV used for infection.

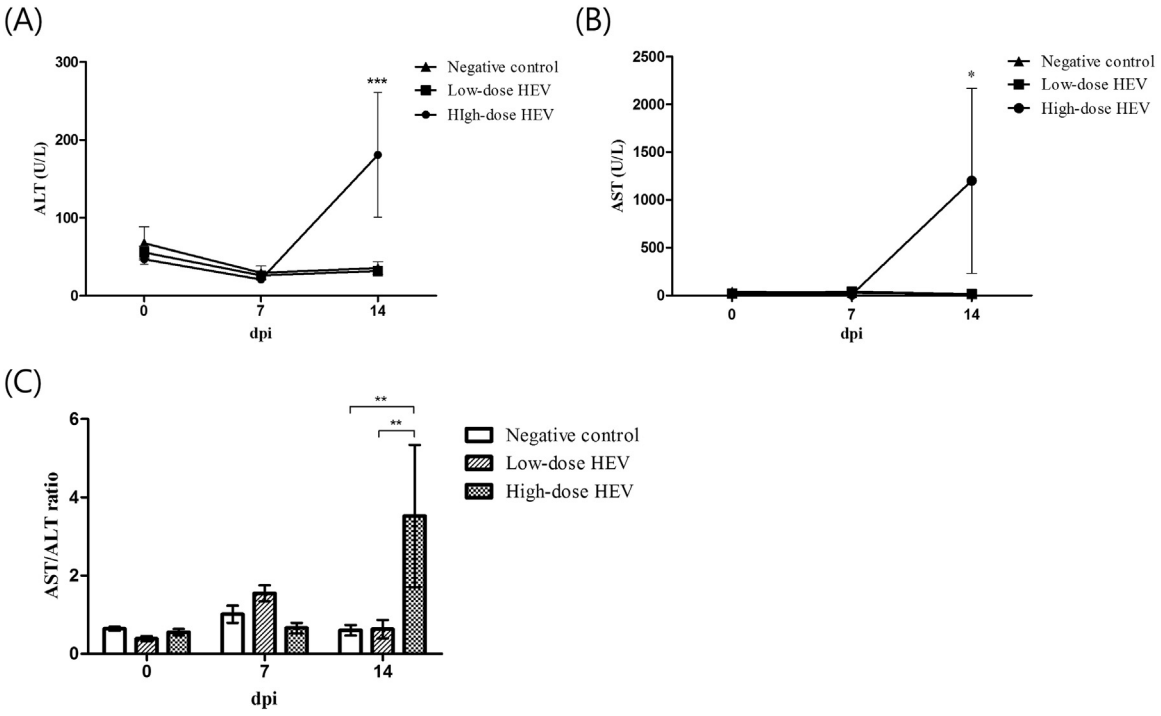

Fig. 1. Determination of ALT and AST concentrations and the AST/ALT ratio (A) ALT and (B) AST levels were measured in serum samples collected from the negative control, low-dose HEV, and high-dose HEV groups 0, 7, and 14 dpi. (C) The AST/ALT ratio was determined in the three groups 0, 7, and 14 dpi. Statistical significance was determined between groups at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

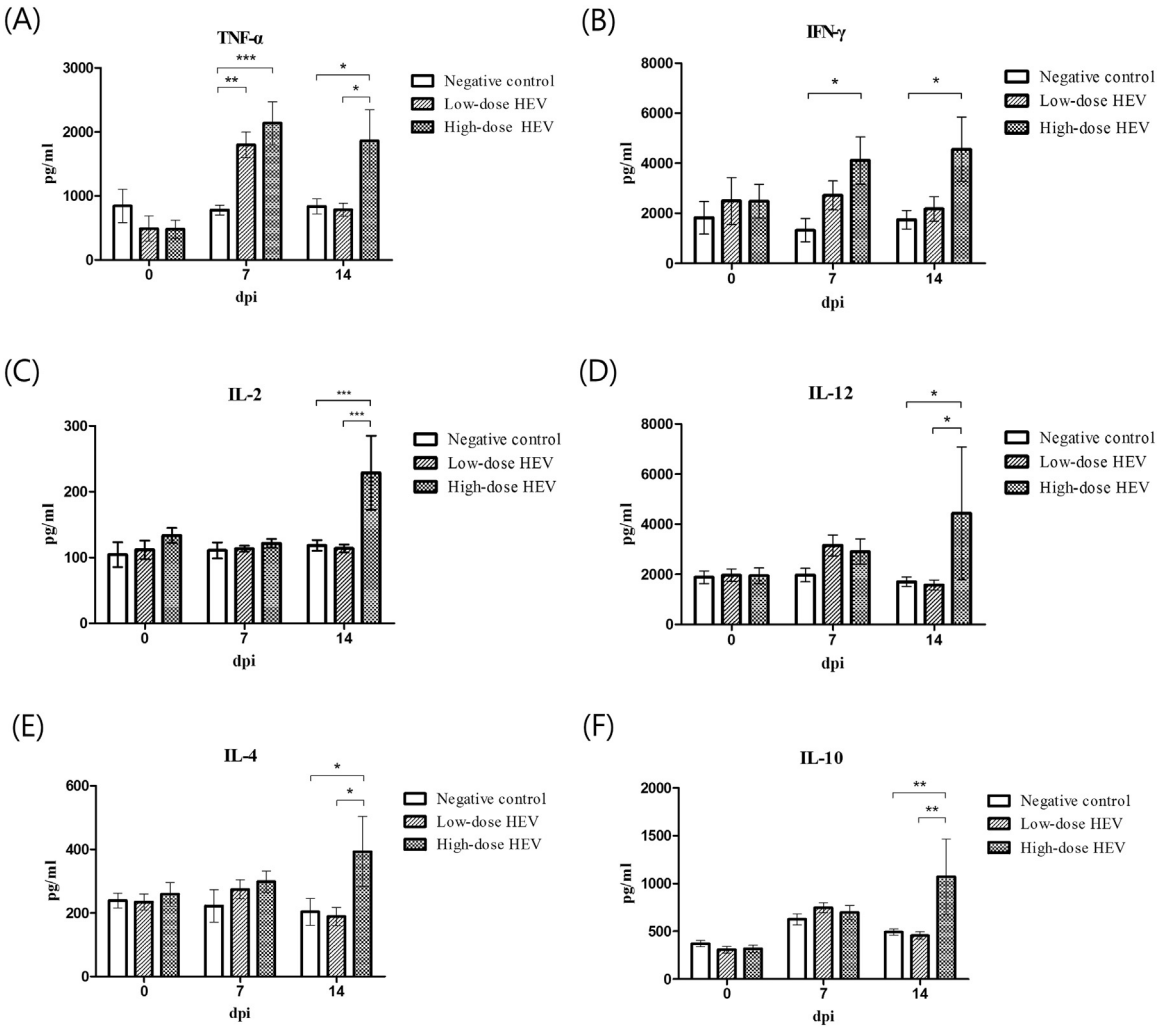

Fig. 2. Determination of cytokine concentrations in serum samples (A) TNF-α, (B) IFN-γ, (C) IL-2, (D) IL-12, (E) IL-4, and (F) IL-10 levels were measured in serum samples collected from the negative control, low-dose HEV, and high-dose HEV groups 0, 7, and 14 dpi. Statistical significance was determined between groups at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Viral replication in rabbits and non-human primates experimentally infected with rHEV is typically verified by observing viremia, fecal shedding of virus, and detection of HEV antigens in tissues, including the liver (Han et al., 2014; Liu et al., 2013; Ma et al., 2010; Xia et al., 2015). We observed viral replication in samples obtained from pregnant rabbits infected with both low and high doses of rHEV. The inoculated virus was intermittently detected in the serum and fecal samples of the rabbits. The HEV antigen was also identified in the liver tissue of infected rabbits concurrently presenting inflammatory lesions. Seroconversion in rabbits and cynomolgus macaques experimentally infected with rHEV usually occurs 4–7 weeks after infection, depending on the doses and viral isolates (Liu et al., 2013; Ma et al., 2010; Xia et al., 2015). In this study, only one rabbit (#14), which received a high dose of HEV, produced IgG against rHEV 14 dpi. This rabbit produced only IgG, and not IgM. In addition, rHEV was not be detected by nested RT-PCR in the rabbit serum before inoculation. Therefore, we concluded that this rabbit may have been infected with the undetectably low titer of rHEV before the experiment was performed. The IgG may have been produced by an anamnestic response after inoculation of high-dose rHEV. The experimental duration may have been too short to induce antibody production in the other HEV-infected rabbits. Taken together, these results confirm the replication of rHEV in pregnant rabbits, which subsequently causes acute hepatitis.

Acute liver failure and high viral load are suggested to cause the adverse maternal and fetal outcomes observed in HEV-infected pregnant women (Borkakoti et al., 2013; Bose et al., 2011; Patra et al., 2007). Elevated levels of ALT and AST in serum samples are a typical indicator of acute viral hepatitis (Giannini et al., 2005). Acute HEV patients, including pregnant women, also have high concentrations of ALT and AST in their sera (Khuroo et al., 2009; Sugitani et al., 2009). The normal ranges of ALT and AST in rabbits are 55–260 U/L and 10–98 U/L, respectively (Daudu et al., 2014). In the present study, all pregnant rabbits infected with low-dose HEV remained in the normal range of ALT and AST 14 dpi. However, the levels of both aminotransferases in most high-dose HEV-infected pregnant rabbits exceeded the normal ranges at 14 dpi, indicating the induction of acute hepatitis. Interestingly, the levels of AST were much higher than those of ALT in high-dose HEV rabbits, with the AST/ALT ratio exceeding 3. High AST/ALT ratios observed in patients suffering from acute viral hepatitis and hepatocellular carcinoma may be a factor contributing to poor prognosis (Changchien et al., 2008; Schiodt et al., 2003), and are also observed in patients suffering from alcoholic hepatitis and chronic hepatitis C (Nyblom et al., 2004; Pohl et al., 2001). Unlike ALT, which is synthesized only in the liver, AST is produced in several organs including the liver, heart, kidney, brain, and uterus (Giannini et al., 2005; Sattler and Furll, 2004). Extrahepatic replication of HEV has been observed in many organs including the spleen, kidney, stomach, intestine, brain, and placenta (Han et al., 2014; Liu et al., 2013; Xia et al., 2015). Therefore, HEV is now considered a pantrophic virus. A high AST/ALT ratio was also observed in type I interferon receptor-deficient mice that

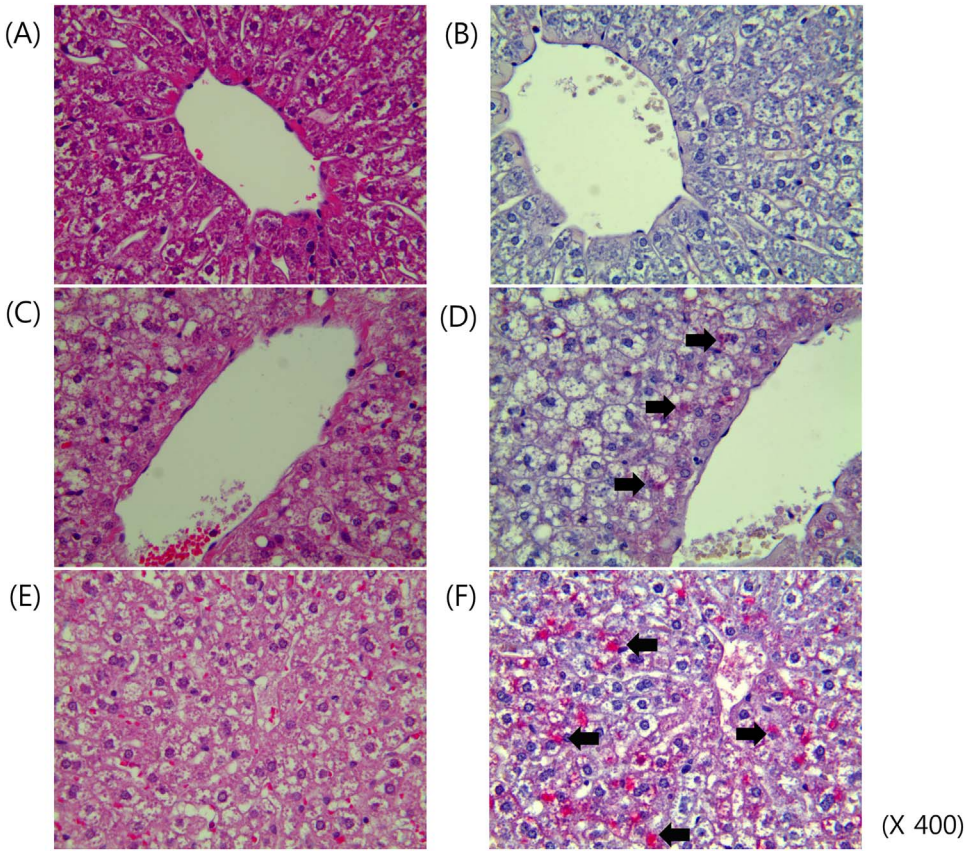

Fig. 3. Histopathological changes and detection of HEV antigen in liver tissues Histopathological changes were determined at 400× magnification following hematoxylin and eosin staining of the liver tissues of representative rabbits from the negative control, low-dose HEV, and high-dose HEV groups, respectively (A, C, and E). HEV antigens were detected by IHC in the liver tissues of representative rabbits in the same groups (B, D, and F). In D and F, arrows indicate HEV antigens.

were infected with Lassa virus, a typical pantrophic virus (Rieger et al., 2013). AST is also an indicator for endometriosis in cows and buffalos (Azawi et al., 2007; Sattler and Furll, 2004). Induction of AST production, and the resultant high AST/ALT ratio in high-dose HEV-infected pregnant rabbits could be a result of damage to extrahepatic tissues, including the uterus. The effect of high uterine AST on fetal outcomes should be examined further.

Severe acute hepatitis and high fetal death rates observed in pregnant women could be mediated by cytokines expressed during HEV infection. High concentrations of TNF-α, IFN-γ, and IL-6 in HEV-infected pregnant women are strongly associated with increased rates of acute hepatitis in the mother and fetal death (Kumar et al., 2014; Salam et al., 2013). In the present study, the expression patterns of Th1 and Th2-type cytokines were determined in serum samples collected at 7 and 14 dpi. The expression of TNF-α was significantly higher 7 dpi in rabbits infected with both low and high doses of HEV than in control rabbits. No cytokines except TNF-α were detected 7 dpi in rabbits infected with HEV. This suggests that high levels of TNF-α during early viral infection might contribute to poor fetal outcomes in pregnant rabbits infected with low-dose HEV, as they did not have elevated levels of ALT or AST. In high-dose HEV infected rabbits, increased TNF-α and IFN-γ coincided with adverse fetal outcomes 7 and 14 dpi. A previous study suggested that high IL-12/IL-10 ratios in HEV-infected pregnant women are a prognostic factor for high fetal and maternal death rates (Bose et al., 2011). However, in the present study, the IL-12/IL-10 ratios in the low- and high-dose HEV infected rabbits were not significantly different from those in the negative control group. Therefore, the IL-12/IL-10 ratio does not seem to be predictive of adverse complications in this rabbit model.

In conclusion, pregnant rabbits infected with high doses of rHEV experienced a higher rate of adverse fetal outcomes than rabbits receiving low doses. High levels of TNF-α, IFN-γ, and AST might be involved in the adverse fetal effects observed in HEV-infected pregnant rabbits.

Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2017.09.020.

References

Ahn, H.S., Park, B.J., Han, S.H., Kim, Y.H., Kim, D.H., Kim, B.S., Lee, J.B., Park, S.Y., Song, C.S., Lee, S.W., Choi, I.S., 2017. Prevalence and genetic features of rabbit hepatitis E virus in Korea. J. Med. Virol.

Azawi, O.I., Omran, S.N., Hadad, J.J., 2007. Clinical, bacteriological, and histopathological study of toxic puerperal metritis in Iraqi buffalo. J. Dairy Sci. 90, 4654–4660.

Bhatia, V., Singhal, A., Panda, S.K., Acharya, S.K., 2008. A 20-year single-center experience with acute liver failure during pregnancy: is the prognosis really worse? Hepatology 48, 1577–1585.

Borkakoti, J., Hazam, R.K., Mohammad, A., Kumar, A., Kar, P., 2013. Does high viral load of hepatitis E virus influence the severity and prognosis of acute liver failure during pregnancy? J. Med. Virol. 85, 620–626.

Bose, P.D., Das, B.C., Kumar, A., Gondal, R., Kumar, D., Kar, P., 2011. High viral load and deregulation of the progesterone receptor signaling pathway: association with hepatitis E-related poor pregnancy outcome. J. Hepatol. 54, 1107–1113.

Burt, S.A., Veltman, J., Hakze-van der Honing, R., Schmitt, H., van der Poel, W.H., 2016. Hepatitis E virus in farmed rabbits, wild rabbits and petting farm rabbits in the Netherlands. Food Environ. Virol. 8, 227–229.

Caruso, C., Modesto, P., Bertolini, S., Peletto, S., Acutis, P.L., Dondo, A., Robetto, S., Mignone, W., Orusa, R., Ru, G., Masoero, L., 2015a. Serological and virological survey of hepatitis E virus in wild boar populations in northwestern Italy: detection of

HEV subtypes 3e and 3f. Arch. Virol. 160, 153–160.
Caruso, C., Modesto, P., Prato, R., Scaglione, F.E., De Marco, L., Bollo, E., Acutis, P.L., Masoero, L., Peletto, S., 2015b. Hepatitis E virus: first description in a pet house rabbit. A new transmission route for human? Transbound. Emerg. Dis. 62, 229–232.
Changchien, C.S., Chen, C.L., Yen, Y.H., Wang, J.H., Hu, T.H., Lee, C.M., Wang, C.C., Cheng, Y.F., Huang, Y.J., Lin, C.Y., Lu, S.N., 2008. Analysis of 6381 hepatocellular carcinoma patients in southern Taiwan: prognostic features, treatment outcome, and survival. J. Gastroenterol. 43, 159–170.
Cossaboom, C.M., Cordoba, L., Dryman, B.A., Meng, X.J., 2011. Hepatitis E virus in rabbits, Virginia, USA. Emerg. Infect. Dis. 17, 2047–2049.
Cossaboom, C.M., Cordoba, L., Sanford, B.J., Pineyro, P., Kenney, S.P., Dryman, B.A., Wang, Y., Meng, X.J., 2012. Cross-species infection of pigs with a novel rabbit, but not rat, strain of hepatitis E virus isolated in the United States. J. Gen. Virol. 93, 1687–1695.
Daudu, O.M., Sani, R.U., Adedibu, I.I., Ademu, L.A., Bawa, G.S., Olugbemi, T.S., 2014. Effect of sweet orange fruit waste diets and acidifier on haematology and serum chemistry of weanling rabbits. J. Vet. Med. 2014 (421382).
Giannini, E.G., Testa, R., Savarino, V., 2005. Liver enzyme alteration: a guide for clinicians. CMAJ 172, 367–379.
Han, J., Lei, Y., Liu, L., Liu, P., Xia, J., Zhang, Y., Zeng, H., Wang, L., Wang, L., Zhuang, H., 2014. SPF rabbits infected with rabbit hepatitis E virus isolate experimentally showing the chronicity of hepatitis. PLoS One 9, e99861.
Huang, C.C., Nguyen, D., Fernandez, J., Yun, K.Y., Fry, K.E., Bradley, D.W., Tam, A.W., Reyes, G.R., 1992. Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology 191, 550–558.
Izopet, J., Dubois, M., Bertagnoli, S., Lhomme, S., Marchandeau, S., Boucher, S., Kamar, N., Abravanel, F., Guerin, J.L., 2012. Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France. Emerg. Infect. Dis. 18, 1274–1281.
Jothikumar, N., Cromeans, T.L., Robertson, B.H., Meng, X.J., Hill, V.R., 2006. A broadly reactive one-step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. J. Virol. Methods 131, 65–71.
Kasorndorkbua, C., Thacker, B.J., Halbur, P.G., Guenette, D.K., Buitenwerf, R.M., Royer, R.L., Meng, X.J., 2003. Experimental infection of pregnant gilts with swine hepatitis E virus. Can. J. Vet. Res. = Revue canadienne de recherche veterinaire 67, 303–306.
Khuroo, M.S., Kamili, S., Khuroo, M.S., 2009. Clinical course and duration of viremia in vertically transmitted hepatitis E virus (HEV) infection in babies born to HEV-infected mothers. J. Viral Hepat. 16, 519–523.
Khuroo, M.S., Teli, M.R., Skidmore, S., Sofi, M.A., Khuroo, M.I., 1981. Incidence and severity of viral hepatitis in pregnancy. Am. J. Med. 70, 252–255.
Kumar, A., Beniwal, M., Kar, P., Sharma, J.B., Murthy, N.S., 2004. Hepatitis E in pregnancy. Int. J. Gynaecol. Obstet. 85, 240–244.
Kumar, A., Devi, S.G., Kar, P., Agarwal, S., Husain, S.A., Gupta, R.K., Sharma, S., 2014. Association of cytokines in hepatitis E with pregnancy outcome. Cytokine 65, 95–104.
Kumar, S., Subhadra, S., Singh, B., Panda, B.K., 2013. Hepatitis E virus: the current scenario. Int. J. Infect. Dis.: IJID: Off. Publ. Int. Soc. Infect. Dis. 17, e228–e233.
Liu, P., Bu, Q.N., Wang, L., Han, J., Du, R.J., Lei, Y.X., Ouyang, Y.Q., Li, J., Zhu, Y.H., Lu, F.M., Zhuang, H., 2013. Transmission of hepatitis E virus from rabbits to cynomolgus macaques. Emerg. Infect. Dis. 19, 559–565.
Ma, H., Zheng, L., Liu, Y., Zhao, C., Harrison, T.J., Ma, Y., Sun, S., Zhang, J., Wang, Y., 2010. Experimental infection of rabbits with rabbit and genotypes 1 and 4 hepatitis E viruses. PLoS One 5, e9160.
Meng, X.J., 2011. From barnyard to food table: the omnipresence of hepatitis E virus and risk for zoonotic infection and food safety. Virus Res. 161, 23–30.
Meng, X.J., Halbur, P.G., Shapiro, M.S., Govindarajan, S., Bruna, J.D., Mushahwar, I.K., Purcell, R.H., Emerson, S.U., 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J. Virol. 72, 9714–9721.
Meng, X.J., Purcell, R.H., Halbur, P.G., Lehman, J.R., Webb, D.M., Tsareva, T.S., Haynes, J.S., Thacker, B.J., Emerson, S.U., 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc. Natl. Acad. Sci. USA 94, 9860–9865.
Nyblom, H., Berggren, U., Balldin, J., Olsson, R., 2004. High AST/ALT ratio may indicate advanced alcoholic liver disease rather than heavy drinking. Alcohol Alcohol 39, 336–339.
Patra, S., Kumar, A., Trivedi, S.S., Puri, M., Sarin, S.K., 2007. Maternal and fetal outcomes in pregnant women with acute hepatitis E virus infection. Ann. Intern. Med. 147, 28–33.
Pohl, A., Behling, C., Oliver, D., Kilani, M., Monson, P., Hassanein, T., 2001. Serum aminotransferase levels and platelet counts as predictors of degree of fibrosis in chronic hepatitis C virus infection. Am. J. Gastroenterol. 96, 3142–3146.
Rieger, T., Merkler, D., Gunther, S., 2013. Infection of type I interferon receptor-deficient mice with various old world arenaviruses: a model for studying virulence and host species barriers. PLoS One 8, e72290.
Salam, G.D., Kumar, A., Kar, P., Aggarwal, S., Husain, A., Sharma, S., 2013. Serum tumor necrosis factor-alpha level in hepatitis E virus-related acute viral hepatitis and fulminant hepatic failure in pregnant women. Hepatol. Res.: Off. J. Jpn. Soc. Hepatol. 43, 826–835.
Sattler, T., Furll, M., 2004. Creatine kinase and aspartate aminotransferase in cows as indicators for endometritis. J. Vet. Med. A Physiol. Pathol. Clin. Med. 51, 132–137.
Schiodt, F.V., Davern, T.J., Shakil, A.O., McGuire, B., Samuel, G., Lee, W.M., 2003. Viral hepatitis-related acute liver failure. Am. J. Gastroenterol. 98, 448–453.
Shalimar, Acharya, S.K., 2013. Hepatitis E and acute liver failure in pregnancy. J. Clin. Exp. Hepatol. 3, 213–224.
Shinde, N., Patil, T., Deshpande, A., Gulhane, R., Patil, M., Bansod, Y., 2014. Clinical profile, maternal and fetal outcomes of acute hepatitis e in pregnancy. Ann. Med. Health Sci. Res. 4, S133–S139.
Smith, D.B., Simmonds, P., International Committee on Taxonomy of Viruses Hepeviridae Study, G., Jameel, S., Emerson, S.U., Harrison, T.J., Meng, X.J., Okamoto, H., Van der Poel, W.H., Purdy, M.A., 2014. Consensus proposals for classification of the family Hepeviridae. J. Gen. Virol. 95, 2223–2232.
Sugitani, M., Tamura, A., Shimizu, Y.K., Sheikh, A., Kinukawa, N., Shimizu, K., Moriyama, M., Komiyama, K., Li, T.C., Takeda, N., Arakawa, Y., Suzuki, K., Ishaque, S.M., Roy, P.K., Raihan, A.S., Hasan, M., 2009. Detection of hepatitis E virus RNA and genotype in Bangladesh. J. Gastroenterol. Hepatol. 24, 599–604.
Tam, A.W., Smith, M.M., Guerra, M.E., Huang, C.C., Bradley, D.W., Fry, K.E., Reyes, G.R., 1991. Hepatitis E virus (HEV): molecular cloning and sequencing of the full-length viral genome. Virology 185, 120–131.
Tsarev, S.A., Tsareva, T.S., Emerson, S.U., Rippy, M.K., Zack, P., Shapiro, M., Purcell, R.H., 1995. Experimental hepatitis E in pregnant rhesus monkeys: failure to transmit hepatitis E virus (HEV) to offspring and evidence of naturally acquired antibodies to HEV. J. Infect. Dis. 172, 31–37.
Wang, Y., Zhang, H., Ling, R., Li, H., Harrison, T.J., 2000. The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J. Gen. Virol. 81, 1675–1686.
Xia, J., Liu, L., Wang, L., Zhang, Y., Zeng, H., Liu, P., Zou, Q., Wang, L., Zhuang, H., 2015. Experimental infection of pregnant rabbits with hepatitis E virus demonstrating high mortality and vertical transmission. J. Viral Hepat. 22, 850–857.
Zhao, C., Ma, Z., Harrison, T.J., Feng, R., Zhang, C., Qiao, Z., Fan, J., Ma, H., Li, M., Song, A., Wang, Y., 2009. A novel genotype of hepatitis E virus prevalent among farmed rabbits in China. J. Med. Virol. 81, 1371–1379.