Vaccine 37 (2019) 5972–5978

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Evaluation of the protective effects of a nanogel-based vaccine against rabbit hepatitis E virus

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ARTICLE INFO

Article history:
Received 27 June 2019
Received in revised form 3 August 2019
Accepted 17 August 2019
Available online 24 August 2019

Keywords:
Rabbit
HEV
Nanogel
Subunit
Vaccine

ABSTRACT

Infection with hepatitis E virus (HEV) has raised serious public health concerns worldwide. In this study, a nanogel-based vaccine encapsulating the capsid protein of rabbit HEV was developed and its protective efficacy was compared with a subunit vaccine. A total of 23 rabbits were divided into 5 groups: (1) negative control (n = 4), (2) positive control (n = 4), (3) nanogel control (n = 5), (4) nanogel vaccine (n = 5), and (5) subunit vaccine (n = 5). Rabbits were vaccinated two times, at weeks 0 and 1, with nanogel and subunit vaccines, respectively, and challenged with rabbit HEV at week 4. By week 11, rabbits vaccinated with the nanogel vaccine produced higher antibodies than those vaccinated with the subunit vaccine. Fecal viral shedding and viremia were identified in rabbits of the positive and nanogel control groups at weeks 6–10. However, there was no viral shedding and viremia in rabbits immunized with both the nanogel and subunit vaccines. Alanine aminotransferase and aspartate aminotransferase levels were not elevated in any rabbit. However, histopathological examination revealed much less hepatic inflammation in rabbits of the nanogel vaccine group compared to the positive and nanogel control groups. Significant increases in IL-12 and IFN–γlevels were identified from rabbits immunized with the nanogel vaccine. Collectively, these results indicate that the newly developed nanogel vaccine induced sufficient immunity leading to complete protection from HEV infection in rabbits. Application of this vaccine should be considered as a preventive measure against HEV infection in other animal species and humans.

1. Introduction

Hepatitis E virus (HEV) belongs to the genus *Orthohepevirus* in the family *Hepeviridae* [1,2]. It has a single-stranded positive-sense RNA genome, about 7.5 kb in length. HEV infection causes an acute self-limiting disease in most cases, but induces 20 to 25% mortality and poor prognosis in pregnant women and patients with underlying chronic liver disease, respectively [3,4]. Recently, chronic infection cases have been reported in immunosuppressed individuals such as solid organ transplant recipients, patients with hematologic malignancies, and patients infected with human immunodeficiency virus [5–8]. Among 8 HEV genotypes, HEV-1 and HEV-2 infect only humans, however, HEV-3 and HEV-4 are known to infect several animals, such as pigs and humans [9,10]. HEV-5 and HEV-6 were isolated from wild boars and HEV-7 and HEV-8 were recently found in camelids [1,11–13]. Zoonotic transmissions of HEV-3, HEV-4, and HEV-7 have been reported from peoples who consumed raw or undercooked pork, camel milk, and sausage made up of pig livers [14–17].

Rabbit HEV is a variant of genotype 3 HEV and is also suspected to be a zoonotic agent [18,19]. Since most animal species infected with HEV do not develop clinical signs of hepatitis [20], studying the pathogenesis of the virus and evaluating the protective efficacy of the vaccines has been difficult. However, it has been reported that rabbits infected with HEV produce viremia, fecal viral shedding, and mild hepatitis lesions [21–23].Therefore, they are regarded as suitable animal models for studying the viral pathogenesis and protective effects of vaccines against HEV infection. Recently, a virus-like particle vaccine, composed of 239 amino acids of the HEV-1 capsid protein, was developed in China for humans [24]. However, other types of vaccines need to be developed using genotype 3 HEV, which is the most prevalent virus in the world.

Nanogel-based techniques have been applied to many scientific research areas, but most studies focus on drug delivery systems

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https://doi.org/10.1016/j.vaccine.2019.08.029
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[25]. Nanogel-based vaccines are known to induce maturation of dendritic cells by strongly stimulating them through a particulate delivery mechanism [26–28]. It has been reported that nanogel-based protein delivery systems can efficiently promote the presentation of antigens to T lymphocytes, which lead to the induction of strong humoral and cellular immune responses [29,30]. Therefore, it would be a reasonable strategy applying nanogel-based vaccines to prevent infectious diseases, including viral disease.

In this study, we developed a nanogel-based vaccine containing the rabbit HEV capsid protein using oligo (ethylene glycol) monomethyl ether methacrylates (OEOMA) and evaluated the protective efficacies of the vaccine against HEV in rabbits.

2. Materials and methods

2.1. Expression of the recombinant rabbit HEV capsid protein

The capsid gene of rabbit HEV was synthesized after codon optimization for bacterial expression. The synthetic HEV capsid gene digested by *Bgl*II and *Hind*III were ligated into a pQE40 expression vector (Qiagen, Germany). The recombinant HEV capsid protein was expressed in M15 competent cells (Qiagen) and purified in 8 M urea buffers following the manufacturer's instructions (Qiagen). The recombinant capsid protein was identified via SDS-PAGE and western blot with a rabbit polyclonal antibody specific to HEV (Sigma-Aldrich, USA). The recombinant capsid protein was used as a subunit vaccine and as a nanogel-based vaccine after encapsulation with nanogel-material.

2.2. Synthesis of nanogel vaccine encapsulating the rabbit HEV capsid protein

For polymer synthesis, 2-bromoisobutyryl bromide (0.25 g, 2.0 mmol) (Sigma-Aldrich, USA) was added into 150 ml dichloromethane (Sigma-Aldrich) with 5 g poly (ethylene glycol) monomethyl ether (PEO5000- OH) (Sigma-Aldrich) and 0.28 g trimethylamine (Sigma-Aldrich), and gently mixed for 16 h at room temperature. Hexane (Sigma-Aldrich) was added for precipitation of PEO5000 – Br after evaporation, and the precipitate was dried in a vacuum container at 25 °C for 24 h. The rabbit HEV capsid protein was mixed in 1.2 ml 2 M guanidine hydrochloride buffer with 1.2 g Oligo (ethylene glycol) monomethyl ether methacrylate (OEOMA300) (Sigma-Aldrich), 65.65 mg PEO5000 – Br, 1.93 mg tris-[(2-pyridyl)methyl]amine (TPMA) (Sigma-Aldrich), 1.48 mg $CuBr_2$ (Sigma-Aldrich) and 90 mg Poly (ethylene glycol) dimethacrylate (PEGDMA) (Sigma-Aldrich, St Louis, MO, USA). Cyclohexane (17.14 g; Sigma-Aldrich) with 857 mg Span® 80 (Sigma-Aldrich) was added to the mixture in an oil bath at 30 °C. The reaction was initiated by adding 0.004 mmol ascorbic acid in the presence of nitrogen gas, then the mixture was exposed to air after 24 h. The product was rinsed in cyclohexane, tetrahydrofuran (THF) (Sigma-Aldrich), and PBS by centrifuging at 15,000 × g at 4 °C for 30 min. The rinsed nanogel was suspended in phosphate-buffered saline (PBS) solution and used as a vaccine capsule. Dynamic light scattering (DLS) analysis was performed using a 90 Plus Particle Size Analyzer (Brookhaven Instruments Corporation) and non-contact (tapping) atomic force microscopy (AFM) was performed with an n-Tracer SPM (Nanofocus).

2.3. Preparation of rabbit HEV

Rabbit HEV (GenBank accession number KY496200) was obtained from rabbit fecal samples. The genomic copy number of the virus was determined by real-time polymerase chain reaction (PCR) as previously reported [31]. The titer of rabbit HEV in the fecal supernatant was adjusted to 10^6 genomic copy number/mL by dilution with 4% bovine serum albumin solution.

2.4. Rabbit immunization, viral challenges, and histological examination

Animal experiments were approved by the Institutional Animal Care and Use Committee of Konkuk University (IACUC No. KU16128). All rabbits were kept in the animal facility of Konkuk Laboratory Animal Research Center at biosecurity level 2. Specific pathogen-free 6-week-old rabbits were obtained from a commercial animal supplier (Orient Bio, Korea). A total of 23 rabbits were divided into five groups: 4, negative control; 4, positive control; 5, nanogel control; 5, nanogel vaccine; and 5, subunit vaccine groups. Rabbits in the negative and nanogel control groups were intramuscularly injected with 1 ml PBS and nanogel solution at weeks 0 and 1, respectively. Rabbits in the two vaccine groups were intramuscularly injected twice, at weeks 0 and 1, with 30 μg of the subunit vaccine and the nanogel vaccine, respectively, without adjuvants. Rabbits in the four groups, except the negative control, were intravascularly challenged at week 4 with the 10^6 genomic copies of rabbit HEV. All rabbits were euthanized at 11 weeks by administration of a mixture of Zoletil (50 mg/kg) and xylazine (5 mg/kg) and the liver samples were collected into 4% formaldehyde solution. The formaldehyde-fixed liver samples were hematoxylin and eosin stained and examined as previously described [32].

2.5. Detection of rabbit HEV RNA

The viral RNA in the fecal and serum samples of rabbits was extracted with the Patho Gene-spin DNA/RNA Extraction Kit (Intron, Korea) according to the manufacturer's instructions. The extracted viral RNA was stored at −70 °C until use. The presence of rabbit HEV from the RNA samples was determined via nested RT-PCR as described previously [23].

2.6. Measurement of anti-HEV antibodies, cytokines, and liver enzymes.

Blood samples were collected from the ear veins of rabbits prior to each vaccination and weekly for 11 weeks. The HEV-specific antibody titers were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Wantai, China). Seroconversion was determined when the anti-HEV titers were higher than the cut-off value (O. D. 0.12) according to the manufacturer's instructions.

The serum concentrations of IL-2, IL-4, IL-10, IL-12, IFN-γ, and TNF-α were determined via commercial ELISA kits, according to the manufacturers' instructions: rabbit IL-2 DuoSet ELISA kit (R&D System, USA), rabbit IL-4 DuoSet ELISA kit (R&D System), rabbit IL-10 ELISA kit (Cusabio, China), rabbit IL-12 ELISA kit (FineTest, China), rabbit TNF-α ELISA kit (Cusabio), and rabbit IFN-γ ELISA kit (Raybio, USA) in duplicates. Serum concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by UV-assay at the Neodin Veterinary Science Institute (Seoul, Korea), according to the International Federation of Clinical Chemistry and Laboratory Medicine protocol, without pyridoxal phosphate activation.

2.7. Statistical analysis

Data were expressed as means ± SEM. The experimental results were analyzed using two-way ANOVA by GraphPad Prism ver. 5.00 (Graphpad Software, USA). Bonferroni multiple-comparisons test was carried out as the post hoc test. The level of statistical significance was set at $P < 0.05$.

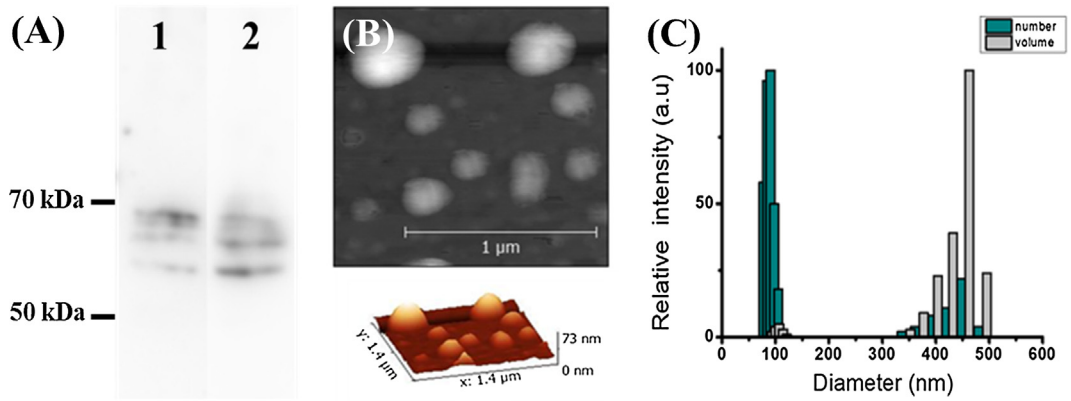

Fig. 1. Production of subunit and nanogel vaccines. (A) Identification of the rabbit HEV capsid proteins by western blot. Lane 1: subunit vaccine composed of the rabbit HEV capsid protein, lane 2: nanogel vaccine containing the rabbit HEV capsid protein encapsulated by nanogel, (B) and (C) Nanogel particle sizes and forms analyzed by DLS and AFM.

3. Results

3.1. Development of HEV capsid subunit and nanogel-based vaccines

Subunit and nanogel encapsulated vaccines each composed of the rabbit HEV capsid protein were generated. The rabbit HEV capsid protein in the two vaccines was detected at 67 kDa by western blot (Fig. 1A). The nanogel particles had circular forms (Fig. 1B) ranging from 80 to 120 nm in size (Fig. 1C). The nanogel encapsulation efficiency was 48.53% (data not shown), and the nanogel vaccine maintained its nano-structure in water and an organic solvent.

3.2. Seroconversion after vaccination and viral challenge

Seroconversion in serum samples collected from all rabbits in the five experimental groups over 11 weeks was determined using ELISA. Rabbits in the negative control did not demonstrate seroconversion during the experimental periods as expected (Fig. 2). When the anti-HEV antibody titers were measured at week 2, all rabbits in the two vaccine groups produced higher titers than the cutoff value (O. D. 0.12) (Fig. 2). After the viral challenge at week 4, the antibody titers of rabbits in the subunit vaccine were dramatically elevated during weeks 5–7, but slowly declined thereafter until week 11 (Fig. 2). However, the anti-HEV antibody titers of the nanogel vaccinated rabbits continuously increased during weeks 5–11 (Fig. 2). Rabbits in the nanogel and positive control groups also produced anti-HEV antibodies after viral challenges (Fig. 2). These results indicate that the nanogel vaccine might have a more durable immunogenic nature than the subunit vaccine.

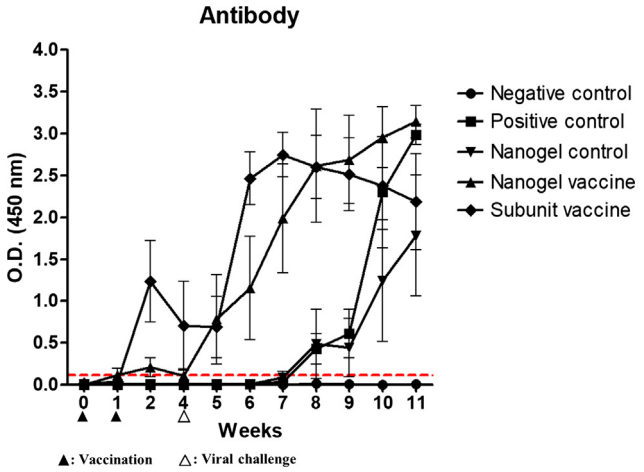

Fig. 2. Determination of anti-HEV antibody titers. Serum samples were collected weekly from all rabbits used in the five experimental groups and anti-HEV antibody titers were determined via ELISA. Black arrow head: vaccination at weeks 0 and 1, white arrow head: virus challenge at week 4, dashed line: cut-off value O. D. 0.12. Anti-HEV titers higher than O. D. 0.12 indicated seroconversion.

3.3. Protective efficacy of the subunit and nanogel-based vaccines

Rabbit HEV RNA in serum and fecal samples of all rabbits was determined via RT-PCR from weeks 0–11. As expected, viral RNA could not be detected in any sample collected from the negative control rabbits (Table 1). Viral RNA could be detected from fecal and/or serum samples collected from rabbits in the positive and nanogel control groups during weeks 6–10 (Table 1). However, viral RNA could not be determined from any serum or fecal sample collected from rabbits that were immunized with the subunit or nanogel vaccine after viral challenges (Table 1). These data indicate that both the subunit and nanogel vaccines induced complete protective immunity against rabbit HEV infections.

3.4. Liver enzymes and histopathological examinations

Both ALT and AST levels of all rabbits in the five experimental groups remained within normal ranges without significant differences among them throughout the experiment (Fig. 3A and B). However, focal and/or scattered chronic inflammatory cells were found in hepatic lobules of all rabbits in the positive and nanogel control groups (Fig. 4B and C). However, the focally distributed inflammatory cells were occasionally observed in the negative control, subunit vaccine, and nanogel vaccine groups (Fig. 4A, D, and E). The average histopathological scores of the negative control, nanogel vaccine, and subunit vaccine groups were 0.5, 0.8, and 1.0, respectively (Table 1). The histopathological scores of the two vaccine groups were much lower than those of the positive (2.0) and nanogel (1.6) control groups (Table 1). These results imply that the nanogel vaccine surely contributed to the less severe inflammatory responses in the liver tissues of the vaccinated rabbits after the viral challenge.

Table 1
Detection of fecal viral shedding and viremia and evaluation of histopathological scores.

Group	Weeks	0	1	2	3	4	5	6	7	8	9	10	11	Histopathological score	Average of score
	No.	F/S[a]	F/S	F/S	F/S	F/S	F/S	F/S	F/S	F/S	F/S	F/S	F/S		
Negative control	1	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0	0.5
	2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1	
	3	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0	
	4	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1	
Positive control	5	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/-	-/-	-/-	-/-	-/-	2	2.0
	6	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	-/-	-/-	2	
	7	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	-/-	1	
	8	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/-	-/-	-/-	-/-	3	
Nanogel vaccine	9	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1	0.8
	10	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	2	
	11	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1	
	12	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0	
	13	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0	
Subunit vaccine	14	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0	1.0
	15	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	1	
	16	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	2	
	17	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	0	
	18	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	2	
Nanogel control	19	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	2	1.6
	20	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	-/-	+/-	-/-	1	
	21	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	+/-	-/-	-/-	3	
	22	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	-/-	-/-	1	
	23	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+/-	-/-	-/-	1	

[a] F: Feces, S: Serum.

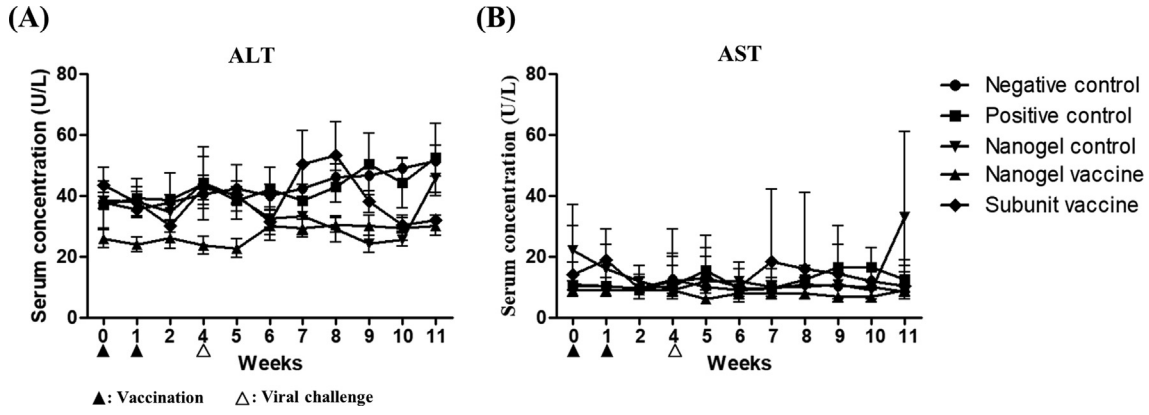

Fig. 3. Determination of serum liver enzymes. Serum concentrations of (A) ALT and (B) AST were determined using commercial kits. Black arrow head: vaccination at weeks 0 and 1, white arrow head: virus challenge at week 4.

3.5. Measurement of serum cytokines

IL-2, IL-4, IL-10, IL-12, IFN-γ, and TNF- α concentrations in serum samples collected for 10 weeks from rabbits in the five experimental groups were determined. No differences in serum concentrations of IL-2, IL-4, IL-10, and TNF-α were observed among rabbits in the five experimental groups (Supplementary Fig. 1). However, the serum concentration of IL-12 increased significantly (P <0.05) at weeks 1 and 2 after vaccination, and at week 8 after viral challenge in rabbits belonging to the nanogel vaccine group when compared with those of the negative control group (Fig. 5A). Serum concentrations of IFN-γ also increased significantly (P <0.05) at weeks 8 and 10 in rabbits that were vaccinated with the nanogel vaccine and challenged with rabbit HEV (Fig. 5B). Rabbits vaccinated with the subunit vaccine did not produce IL-12 and IFN-γ in their serum samples after vaccination and viral challenge (Fig. 5A and B). These results indicate that the nanogel vaccine induced stronger Th1 type immune response in the vaccinated rabbits than the subunit vaccine.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2019.08.029.

4. Discussion

HEV replication and pathogenesis are partially understood because of difficulty in establishing *in vitro* cell culture systems and *in vivo* animal models for viral infections. Most human patients resolve HEV infections after a short acute phase [4]. Recently, chronic Hepatitis E cases have been reported in immunosuppressed patients, such as solid organ transplant recipients or people infected with the human immunodeficiency virus [5–8]. HEV have been isolated from rabbits in several nations, including Korea [18,19,33]. Rabbits infected with HEV show no detectable or very mild symptoms. However, pregnant rabbits produced clinical signs, such as abortion and acute hepatitis, in our previous study [21]. Rabbits infected with HEV also provided evidences of fecal viral shedding and viremia. Therefore, rabbits are considered as

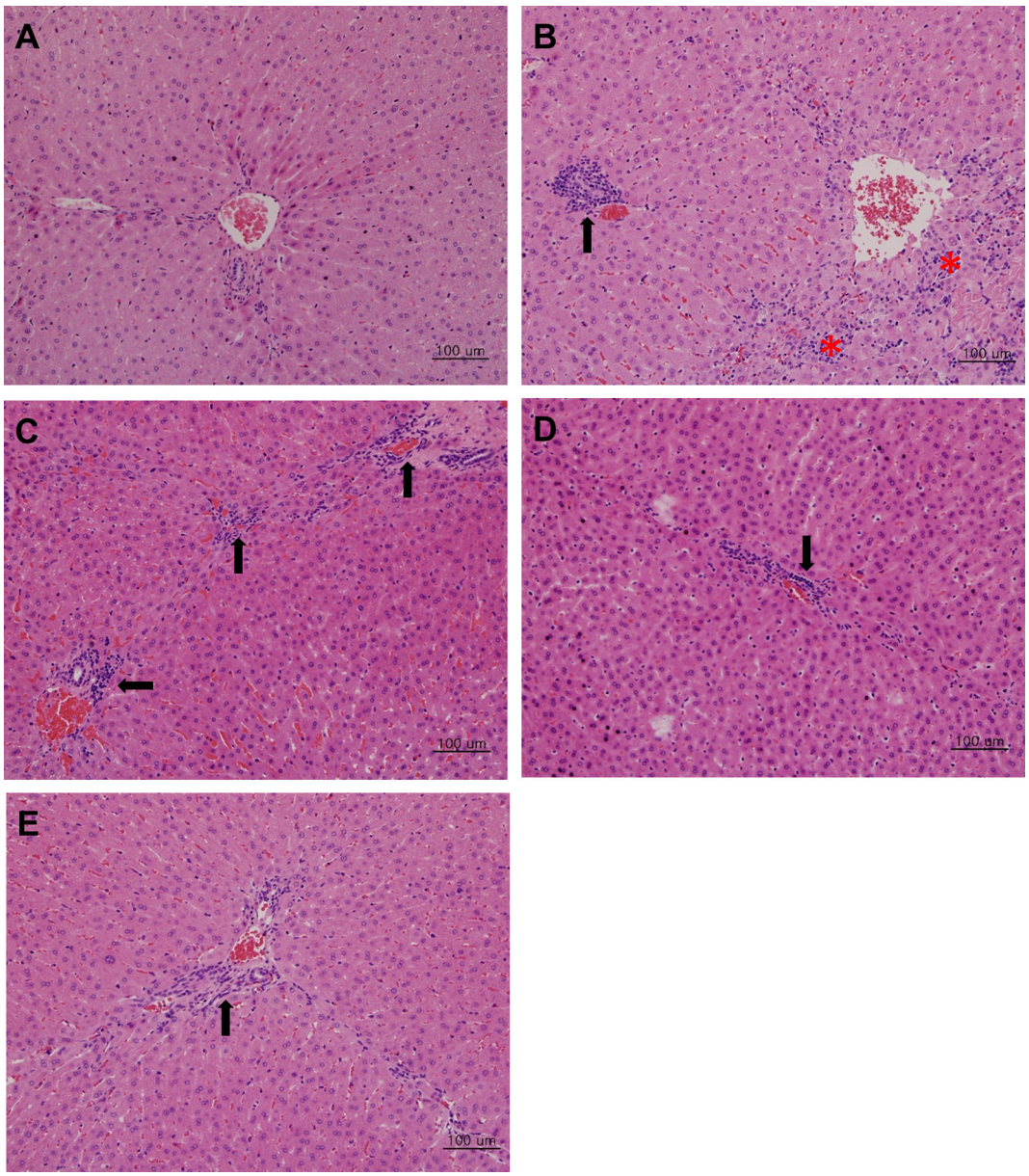

Fig. 4. Representative images of histopathological lesions in rabbit livers. (A) Negative control rabbit (No. 3) with no histopathologic lesions; (B) Positive control rabbit (No. 8) with chronic inflammatory cells distributed with focal (arrow) or scattered (asterisks) patterns in hepatic lobules; (C) Nanogel control rabbit (No. 21) with chronic inflammatory cells with multifocal (arrows) distribution in hepatic lobules; (D) Nanogel vaccine rabbit (No. 10) with chronic inflammatory cells with focally distributed (arrow) patterns in hepatic lobules; and (E) Subunit vaccine rabbit (No. 16) with chronic inflammatory cells focally distributed (arrow) patterns in hepatic lobules. H&E stain. Scale bar = 100 μm.

appropriate animal models for studying HEV pathogenesis and evaluating HEV vaccine efficacy.

The nano-technologies widely used for drug delivery systems have been applied for the induction of specific and strong immune responses *in vitro* and *in vivo* [26–30]. For example, nanogels decorated with ligands for immune cell receptors were used to induce dendritic cell (DC) maturation and stimulation [26,30]. The polyelectrolyte-based nanogel vaccine promoted the uptake of antigens into bone marrow DC and preferentially induced Th1-specific immunity [29,34]. In this study, we developed OEOMA-based HEV nanogel vaccine and evaluated its protective efficiency by comparing with a subunit vaccine in rabbits. Both the subunit and nanogel vaccines showed complete protective effects against HEV infections. Rabbits vaccinated with both vaccines produced neither viremia nor fecal viral shedding. Rabbits vaccinated with the subunit vaccine more rapidly produced anti-HEV antibodies than those vaccinated with the nanogel vaccine. Their antibody titers were also sharply increased after viral challenge. However, their antibodies declined more quickly than those produced in rabbits vaccinated with the nanogel vaccine. Antibody titers for rabbits vaccinated with the nanogel vaccine remained continuously elevated throughout the study. These results imply that the HEV capsid antigen encapsulated in the nanogel might become released more slowly than the capsid antigen contained in the subunit vaccine.

After vaccination of rabbits with both vaccines, the expression of six cytokines (IL-2, IL-4, IL-10, TNF- α, IL-12, and IFN-γ) in serum samples of all rabbits were measured to determine inflammation, Th1, or Th2 immune response. Among the six cytokines, only IL-12 and IFN-γ were significantly increased in rabbits immunized with the nanogel vaccine. IL-12 and IFN-γ are known as typical Th1-type cytokines which promote cellular immunity [35,36].

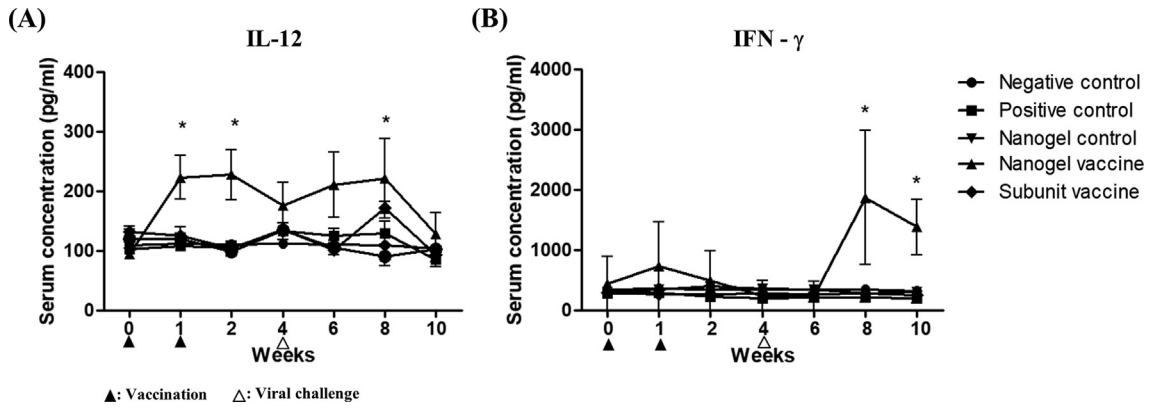

Fig. 5. Determination of cytokine levels in rabbit serum samples. Concentrations of (A) IL-12 and (B) IFN-γ were determined in serum samples collected from all rabbits in the five experimental groups. Black arrow head: vaccination at weeks 0 and 1, white arrow head: virus challenge at week 4. Significance was determined at * P <0.05.

Th1-type-mediated immune responses are known to be critical in the clearance of other hepatitis viruses [37,38]. Therefore, the clearance of HEV in rabbits vaccinated with the nanogel vaccine might be mediated by both humoral and cellular immune responses. In contrast, the subunit vaccine did not induce significant production of any kind of Th1 or Th2-type cytokines examined in this study. Viral clearances, only by humoral immune response, have been proven by several studies [24,39]. Therefore, it could be assumed that the clearance of HEV in rabbits vaccinated with the subunit vaccine might be achieved by humoral immunity. No significant difference was found in the expression of other cytokines (IL-2, IL-4, IL-10, and TNF- α) in all rabbits used in this experiment (Supplementary Fig. 1).

The serum concentration of liver enzymes (ALT and AST) in all rabbits of the five experimental groups were not elevated. It is well known that clinical signs of acute hepatitis were not produced in animals including rabbits except for pregnant rabbits infected with HEV [20–23]. HEV infection induced severe adverse effects in the pregnant rabbits, such as abortion, stillbirth, and reduced offspring numbers [21,23]. They also showed high levels of AST and ALT, demonstrating severe liver damage due to HEV infections [21,23]. Unlike our results in this study, a few studies demonstrated slight elevations in liver enzyme concentrations in rabbits infected with HEV [22]. This discrepancy could be attributed to differences in viral doses, viral strains, and experimental conditions. In contrast with serum liver enzyme levels, histopathological lesions, such as focal and scattered inflammations, were obviously reduced, especially in rabbits immunized with the nanogel vaccine. Their inflammatory scores in hepatic tissues were similar with those determined in negative control rabbits. These results suggest that the nanogel vaccine played an important role in protecting rabbits from HEV challenges.

In this study, we developed a new HEV vaccine based on nanogel technology and proved its protective efficiency against rabbit HEV. The nanogel vaccine produced a prolonged humoral and stronger Th1-type immune responses than the subunit vaccine. Both humoral and cellular immune responses induced by the nanogel vaccine might be involved in HEV elimination from rabbits and mitigate inflammations in their liver tissues. We anticipate that this new nanogel vaccine platform would be applicable for the development of several human and animal vaccines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was supported by the Ministry of Health & Welfare, Republic of Korea.

Funding

This study was supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI18C1836).

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