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# Subcutaneous Toxicity Evaluation of a Combination Vaccine against Hantaan and Puumala Viruses in Rats for 4 Weeks

Su-Hae Lee, Eun-Yong Jung, Kyong-Ok No, Ji-Soon Sin, Chang-Nam Ahn[1], Dae-Joong Kim, Sang-Yoon Nam and Jong-Koo Kang*

*College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763 and*
*[1]Research Institute, Green Cross Co. Ltd., 227 Kugal-ri,*
*Kiheung-eup, Yongin-city 449-900, Korea*
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***ABSTRACT*** *: Hantaan (HTNV) and Puumala (PUUV) viruses cause hemorrhagic fever with renal syndrome in human. In the present study, the repeated dose toxicity of the HTNV-PUUV combination vaccine was evaluated in Sprague-Dawley rats. Animals were injected subcutaneously for 28 days with dosages of 0, 0.017, 0.17 and 1.7 dose/kg body weight per day, respectively. No any significant changes of body weight, food and water consumptions were shown. There were no death and clinical findings during the experimental period. In both male and female rats, there were not significant changes in hematological and serum biochemical analysis, urinalysis, and ophthalmoscopic and histopathological examinations. These results indicate that the HTNV-PUUV combination vaccine may have no toxic effects and no observed adverse effect level (NOAEL) may be over 1.7 dose/kg/day at subcutaneous route in rats.*

***Key Words*** *: Repeated dose toxicity, HTNV-PUUV combination vaccine, Rats*

## I. INTRODUCTION

Hantaviruses, forming a separate genus of the Bunyaviridae family, are enveloped viruses with negative-sense single-stranded RNA genomes and are the causative agents of several human diseases with similar clinical symptoms, commonly referred to as hemorrhagic fever renal syndrome (HFRS) on the Eurasian continent and currently hantavirus pulmonary syndrome (HPS) on the American continent (Nichol *et al.*, 1993; Schmaljohn *et al.*, 1985). Several serotypes have been described from human and at least five are pathogenic to human: i) the prototype Hantaan virus (HTNV) which is mainly documented in Asia, ii) Seoul virus (SEOV) with a world wide distribution, iii) Puumala virus (PUUV) which is predominantly endemic throughout Europe, iv) Dobrava virus (DOBV) which is recognized in eastern and central Europe, and v) Sin Nombre virus (SNV) which is documented in the Americas (Avsic-Zupanc *et al.*, 1995; Hjelle *et al.*, 1994; Lee *et al.*, 1978). Each virus has a specific rodent host and transmission of virus to human occurs via aerosolized urine, feces, saliva and excreta of infected animals: i) the striped field mouse *Apodemus agrarius* for HTNV, ii) the commensal rats *Rattus norvegicus* and *rattus* for SEOV, iii) the bank vole *Clethrionomys glareolus* for PUUV, iv) the yellow-necked field mouse *Apodemus flavicollis* for DOBV and v) the deer mouse *Peromyscus maniculatis* for SNV (Meyer *et al.*, 2000).

Lee *et al.* (1989) developed a formalin-inactivated HTNV vaccine (Hantavax™) derived from suckling mouse brain available commercially in 1991 in Korea. HTNV 84/105 strain isolated from blood of a HFRS patient was used as the vaccine seed virus in Vero-E6 cells. In addition, it was confirmed in HRFS patients infected with PUUV in Korea. PUUV exists not only in Europe but also in Asia, eastern part of Russia and Japan

*To whom correspondence should be addressed.
List of Abbreviations : HFRS, hemorrhagic fever renal syndrome; HPS, hantavirus pulmonary syndrome; HTNV, Hantaan virus; SEOV, Seoul virus; PUUV, Puumala virus; DOBV, Dobrava virus; SNV, Sin Nombre virus; WBC, white blood cell; RBC, red blood cell; Hgb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; PT, prothrombin time; APTT, activated partial thromboplastin time; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; BUN, blood urea nitrogen; GGT, γ-glutamyl transpeptidase; LDH, lactate dehydrogenase; TG, triglyceride.

(Horing *et al.*, 1996; Kariwa *et al.*, 1995). Therefore, an effective combination vaccine against HTNV and PUUV infection has been needed urgently. Recently, Korea Green Cross Co. (Yongin, Korea) developed a formalin inactivated PUUV vaccine derived from suckling hamster brain to prevent PUUV infection and a HTNV-PUUV combination vaccine to prevent HFRS caused by HTNV and PUUV infection in the world. The PUUV K27 strain isolated from a HFRS patient in Ufa, Bashikiria was used as the seed virus (Lee *et al.*, 1997).

The aim of this study was to evaluate the repeated dose toxicity of the HTNV-PUUV combination vaccine in rats according to the guideline for toxicity tests of drugs proposed by Korea Food and Drug Administration (1999).

## II. MATERIALS AND METHODS

### 1. Test substances

The HTNV-PUUV combination vaccine (Yongin, Korea) was diluted with phosphate buffered saline (PBS). Sterile PBS was used as the carrier and control. The test substance was acceptable to the quality assurance criteria and stored at 4°C in the dark before use. The lot number of the test substance used for this study was VI-005. Other chemicals and reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, U.S.A.).

### 2. Animals and maintenance

Five-week-old specific pathogen-free Sprague-Dawley (SD) rats of both sexes were purchased from Dae Han Laboratory Animal Research Center Co., Ltd. (Eumsung, Korea). Animals were housed in an animal room maintained with 12 hrs (07:00~19:00) light-dark cycle at a constant temperature of 23±2°C and a relative humidity of 50±5%, and were used after 1 week acclimation period. Forty males, weighing 200 to 220 g, and 40 females, weighing 156 to 176 g, were employed in this study. All animals were housed in polycarbonate cages (260 W×420 L× 180 H mm) and given a commercial solid diet (Samyang Feed Co., Ltd., Wonju, Korea) and tap water *ad libitum*.

### 3. Experimental design

Four study groups, each consisting of 10 male and 10 female SD rats, were received subcutaneously the vaccine at doses of 0.017, 0.17 and 1.7 dose/kg body weight in PBS daily for four weeks. One dose of HTNV-PUUV combination vaccine contains both 5,120 units/ELISA of HTNV and PUUV antigen in 1.0 ml. High dose level (1.7 dose/kg) is equivalent to 100-fold of a dosage (0.017 dose/kg) used clinically in human when the body weight is assumed to be 60 kg. These doses were selected from the results of single dose toxicity test (data not shown). The administration volume was daily calculated according to the increasing body weight (2 ml/kg body weight).

### 4. Observations and examinations

The clinical signs were observed at least twice daily, before and after injection in all animals during the experimental period. The body weight, food consumption and water intake were measured once a week during the treatment period. Opthalmoscopic examination was conducted to all rats at the final week of experiment. Ocular region and the anterior portion of the eye were observed with an opthalmoscope (RC-2; Kowa, Japan). Then, the pupil was dilated with a mydriatic (Midorin P; Japan) and fundus was observed using an ophthalmoscope. Urinalyses were performed using the fresh samples collected in 24 hrs of the final treatment, and were measured specific gravity, pH, leukocyte, nitrite, protein, glucose, ketone, urobilinogen, bilirubin, blood and hemoglobin using urinalysis stick (Comber-$^{10}$test®; Boehringer Mannheim, Germany). Blood was collected from posterior vena cava in EDTA-contained tubes. Hematological parameters including white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hgb) concentration, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet (PLT) count were examined using a hematological autoanalyzer (Celltac α; Nihon Koden, Japan). WBC differential counts were determined from blood smears stained with Wright-Giemsa. The whole blood smear samples were also stained with new methylene blue for the quantitative determination of reticulocytes. Pro-

thrombin time (PT) and activated partial thromboplastin time (APTT) were determined using a blood coagulation analyzer (ACL 100; Instrumentation Laboratory, Italy) in plasma samples treated with 3.13% sodium citric acid. Serological parameters such as alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), cholesterol, glucose, total protein (T-protein), albumin, blood urea nitrogen (BUN), calcium, creatine kinase, creatinine, γ-glutamyl transpeptidase (GGT), inorganic phosphate, lactate dehydrogenase (LDH), total bilirubin (T-bilirubin), triglyceride (TG) and A/G ratio were analyzed with a automatic blood chemistry analyzer (Express Plus; Chiron, USA). Chloride, sodium, and potassium concentrations were also determined using an automatic electrolyte analyzer (Chiron, USA). The major organs and tissues including testes, epididymis, prostate gland, ovary, uterus, pituitary gland, brain, thymus, heart, lung, liver, spleen, kidney, and adrenal, thyroid and submaxillary glands were weighed, and fixed in 10% neutral buffered formalin (except the eye and Harderian gland, which were fixed in a mixed solution of formaldehyde-glutaraldehyde before formalin fixation) for histopathological examination. The fixed tissues were trimmed and embedded in in paraffin prior to sectioning at 4 μm with a microtome (Leica, Germany), and stained with hematoxylin and eosin.

### 5. Statistical analysis

All results were represented as mean±S.D. and analyzed using analysis of variance procedure (ANOVA) with *post hoc* analysis by Dunnett's *t*-test. A probability level of $p<0.05$ and $p<0.01$ was taken to indicate a significant difference between the control group and the experiment groups.

## III. RESULTS

### 1. General observation

During the study, there was no effect on mortality. All animals survived to the end of experimental period. No notable clinical signs were observed among controls and the groups given the vaccine throughout the dosing period. No other vaccine-related signs were observed in any of the groups.

### 2. Body weight, food consumption and water intake

Mean body weight changes of male and female rats during the experiment were shown in Table 1. Significant changes in body weight were not observed throughtout the dosing period by the vaccine. Food consumption and water intake were not affected by the vaccine (data not shown).

### 3. Ophthalmoscopy and urinalysis

In ophthalmoscopy and urinalysis (Table 2) tests, no vaccine-related changes were observed throughout the treatment periods.

### 4. Hematological analysis (Table 3)

In male rats, significant decreases in MCH ($p<0.05$)

**Table 1.** Changes of mean body weights in the rats injected subcutaneously with the HTNV-PUUV combination vaccine for 4 weeks (Unit : g)

| Sex | Dose (dose/kg) | Days after injection | | | | |
|---|---|---|---|---|---|---|
| | | 0 | 7 | 14 | 21 | 27 |
| Male | 0 | 213.2±6.94 | 228.0±12.51 | 265.8±8.87 | 302.9±11.67 | 333.8±14.50 |
| | 0.017 | 210.6±8.80 | 222.9±13.93 | 260.0±12.79 | 301.5±16.81 | 332.6±27.41 |
| | 0.17 | 210.2±9.31 | 229.9±11.19 | 262.1±7.94 | 307.6±14.43 | 331.9±23.83 |
| | 1.7 | 211.3±8.96 | 230.2±7.33 | 260.6±9.11 | 302.4±19.49 | 327.1±26.99 |
| Female | 0 | 166.7±9.99 | 180.5±10.17 | 199.7±10.88 | 210.7±12.59 | 224.2±15.62 |
| | 0.017 | 166.7±9.04 | 186.9±11.32 | 204.3±12.73 | 215.7±10.46 | 225.8±13.41 |
| | 0.17 | 166.3±9.79 | 180.8±10.81 | 197.2±10.94 | 206.0±10.97 | 216.4±11.68 |
| | 1.7 | 166.8±6.12 | 179.1±12.36 | 196.1±11.97 | 207.8±13.31 | 217.8±14.48 |

Values were expressed as mean±S.D. (n=10).
No significant differences were found between the control and treated groups.
HTNV : Hantaan virus; PUUV : Puumala virus.

**Table 2.** Urinalyses in the rats injected subcutaneously with the HTNV-PUUV combination vaccine for 4 weeks

| Item | Sex | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|---|
| | dose/kg | 0 | 0.017 | 0.17 | 1.7 | 0 | 0.017 | 0.17 | 1.7 |
| | No. of animal | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Specific gravity | 1.005 | 4 | 0 | 0 | 0 | 6 | 0 | 0 | 4 |
| | 1.010 | 4 | 6 | 2 | 5 | 1 | 0 | 0 | 0 |
| | 1.015 | 0 | 2 | 4 | 3 | 0 | 0 | 2 | 2 |
| | 1.020 | 2 | 2 | 4 | 1 | 3 | 6 | 8 | 4 |
| | 1.025 | 0 | 0 | 0 | 1 | 0 | 4 | 0 | 0 |
| pH | 5.0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| | 6.0 | 0 | 0 | 0 | 1 | 3 | 2 | 4 | 2 |
| | 7.0 | 4 | 2 | 8 | 3 | 7 | 6 | 4 | 2 |
| | 8.0 | 6 | 8 | 2 | 5 | 0 | 0 | 2 | 6 |
| | 9.0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| Leukocytes (leuko/μl) | negative | 10 | 10 | 8 | 6 | 10 | 6 | 6 | 10 |
| | 10~25 | 0 | 0 | 2 | 4 | 0 | 2 | 2 | 0 |
| | 75 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 0 |
| Protein (mg/dl) | negative | 2 | 6 | 5 | 2 | 3 | 5 | 5 | 4 |
| | 30 | 8 | 4 | 5 | 8 | 7 | 5 | 5 | 6 |
| Blood (RBC/μl) | negative | 5 | 2 | 4 | 8 | 5 | 0 | 0 | 6 |
| | 5~10 | 1 | 6 | 0 | 2 | 3 | 6 | 0 | 0 |
| | 50 | 4 | 2 | 4 | 0 | 2 | 4 | 4 | 2 |
| | 250 | 0 | 0 | 2 | 0 | 0 | 0 | 6 | 2 |

HTNV : Hantaan virus; PUUV : Puumala virus.

and increases in APTT ($p<0.05$) were observed in the 1.7 dose-treated group. However, no significant changes in the numbers of WBC and all differential leukocytes, and the number and percent of reticulocytes were observed in other rats received the various doses of vaccine.

In female rats, significant changes in WBC ($p<0.05$), MCV ($p<0.05$) and MCHC ($p<0.01$) were observed in the 1.7 dose-treated group. Also, the significant changes in RBC, Hgb, Hct and MCHC in the 0.17 dose-treated group, and WBC, RBC, Hgb and Hct in the 0.017 dose-treated group were observed at $p<0.01$. However, PT and APTT values were in normal range.

### 5. Blood biochemical analysis (Table 4)

In male rats, significant changes of LDH ($p<0.01$), BUN ($p<0.05$), creatinine ($p<0.05$) and T-protein ($p<0.01$) were observed in the 1.7 dose-treated group. In 0.17 dose-treated group, LDH level was significantly decreased ($p<0.01$), while other biochemical parameters were not changed.

In female rats, no significant changes were observed in the blood chemistry parameters, although $K^+$ level was significantly increased in the 0.17 dose-treated group ($p<0.05$).

### 6. Organ weight (Table 5)

In male rats, absolute and relative weights of most organs were not changed except submaxillary gland ($p<0.05$) in the 1.7 dose-treated group.

In female rats, absolute weight of left ovary ($p<0.05$) in the 1.7 dose-treated group, and brains in 1.7 ($p<0.05$), 0.17 ($p<0.01$) and 0.017 dose-treated groups ($p<0.05$) were significantly changed. Only relative weight of spleen ($p<0.05$) was significantly decreased in the 1.7 dose-treated group.

### 7. Gross postmortem findings

In the autopsy of animals at the end of experiment, unusual findings were not observed.

### 8. Histopathological findings

As shown in Table 6, cell infiltraion of renal interstitial tissue was microscopically observed in rats, 1 male at the 0.17 dose/kg group or control groups of both sex, respectively. Vacuolation of renal tubules

**Table 3.** Hematological analyses in the rats injected subcutaneously with the HTNV-PUUV combination vaccine for 4 weeks

| Sex | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| Dose (dose/kg) | 0 | 0.017 | 0.17 | 1.7 | 0 | 0.017 | 0.17 | 1.7 |
| RBC ($10^6/\mu l$) | 7.5±0.68 | 7.5±0.22 | 7.7±0.59 | 7.8±1.46 | 7.0±0.21 | 7.6±0.51** | 7.5±0.43** | 7.3±0.72 |
| Hgb (g/) | 15.5±1.30 | 15.2±0.61 | 15.9±1.18 | 16.4±2.28 | 14.6±0.35 | 16.0±0.71** | 16.0±0.63** | 15.4±1.55 |
| Hct (%) | 41.8±3.94 | 41.5±1.65 | 42.9±3.29 | 44.9±5.78 | 41.0±0.94 | 44.9±1.91** | 44.0±1.87** | 41.8±4.29 |
| MCV (fl) | 55.9±1.85 | 55.5±1.58 | 55.6±1.43 | 55.2±1.32 | 58.8±1.23 | 59.2±1.99 | 58.5±1.43 | 57.3±1.16* |
| MCH (pg) | 20.7±0.55 | 20.4±0.62 | 20.7±0.54 | 20.2±0.62* | 21.0±0.46 | 21.2±0.61 | 21.2±0.66 | 21.0±0.22 |
| MCHC (g/dl) | 37.1±0.66 | 36.7±0.37 | 37.2±0.25 | 36.6±1.00 | 35.7±0.39 | 35.7±0.42 | 36.5±0.72** | 36.7±0.48** |
| PLT ($\times 10^3/\mu l$) | 781.0±119.8 | 794.0±51.6 | 831.9±100.5 | 765.4±140.3 | 721.5±63.9 | 820.4±81.6 | 712.4±207.2 | 786.0±8.3 |
| Reticulocyte (%) | 1.08±0.136 | 1.06±0.155 | 1.09±0.189 | 1.13±0.189 | 1.07±0.152 | 1.05±0.169 | 1.09±0.206 | 1.03±0.08 |
| PT (sec) | 14.7±0.84 | 14.5±0.69 | 14.3±0.7 | 14.3±0.72 | 14.7±0.89 | 14.2±0.42 | 14.5±0.42 | 14.2±0.77 |
| APTT (sec) | 19.4±2.58 | 19.6±2.96 | 21.4±1.8 | 21.6±1.8* | 21.4±1.58 | 21.6±0.95 | 21.7±1.15 | 21.0±1.67 |
| WBC ($\times 10^3/\mu l$) | 9.9±2.63 | 8.9±2.94 | 11.4±2.36 | 10.3±4.43 | 6.6±1.27 | 8.5±1.61** | 6.7±1.33 | 7.9±1.54* |
| Neutrophil | | | | | | | | |
| Band (%) | 1.9±0.66 | 2.1±0.66 | 1.9±0.88 | 1.8±0.67 | 1.7±0.67 | 1.85±0.63 | 1.7±1.03 | 1.3±0.71 |
| Segmented (%) | 3.95±0.9 | 3.4±0.97 | 3.1±0.77 | 3.0±0.82 | 3.75±1.03 | 3.55±0.6 | 2.8±1.18 | 3.35±0.85 |
| Eosinophil (%) | 0.25±0.35 | 0.3±0.42 | 0.25±0.35 | 0.3±0.35 | 0.3±0.26 | 0.3±0.42 | 0.25±0.35 | 0.25±0.26 |
| Basophil (%) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lymphocyte (%) | 91.5±1.35 | 91.83±1.23 | 92.5±0.91 | 92.75±1.01 | 92.2±1.16 | 92.15±1.4 | 93.65±2.29 | 93.15±0.94 |
| Monocyte (%) | 4.8±1.48 | 4.9±1.66 | 2.25±0.72 | 2.15±0.88 | 2.05±0.6 | 2.3±0.71 | 1.6±0.7 | 1.95±0.8 |

Values were expressed as mean±S.D. (n=10).
Significantly different from control at * $p<0.05$ or ** $p<0.01$.
HTNV : Hantaan virus; PUUV : Puumala virus.

**Table 4.** Blood biochemical analyses in the rats injected subcutaneously with the HTNV-PUUV combination vaccine for 4 weeks

| Sex | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|
| Dose (dose/kg) | 0 | 0.017 | 0.17 | 1.7 | 0 | 0.017 | 0.17 | 1.7 |
| ALT (U/l) | 36.4±8.93 | 30.8±7.57 | 35.4±7.75 | 34.4±13.1 | 26.2±4.34 | 24.8±2.35 | 26.0±8.23 | 24.2±4.8 |
| AST (U/l) | 207.2±40.8 | 206.9±47.2 | 186.2±42.7 | 173.0±47.6 | 185.2±10.0 | 182.2±11.9 | 183.2±18.8 | 177.6±19.3 |
| ALP (U/l) | 361.1±126.9 | 308.6±117.4 | 303.2±51.9 | 303.7±83.8 | 233.3±21.9 | 220.9±36.7 | 210.3±35.2 | 224.6±35.9 |
| Cholesterol (mg/dl) | 73.4±18.6 | 66.3±14.6 | 73.8±13.4 | 64.2±10.9 | 61.9±25.5 | 63.2±24.1 | 54.0±16.9 | 58.0±19.0 |
| Glucose (mg/dl) | 86.9±15.1 | 79.2±15.1 | 86.4±8.4 | 85.9±13.2 | 80.1±12.9 | 81.2±10.5 | 76.4±12.3 | 84.3±15.7 |
| T-Protein (g/dl) | 5.9±0.53 | 5.6±0.5 | 5.8±0.85 | 6.2±0.64* | 5.6±0.59 | 5.8±0.33 | 5.9±0.36 | 5.7±0.52 |
| Albumin (g/dl) | 3.5±0.28 | 3.4±0.25 | 3.8±0.79 | 3.6±0.26 | 3.2±0.28 | 3.3±0.21 | 3.3±0.45 | 3.2±0.27 |
| BUN (mg/dl) | 19.8±2.9 | 18.1±2.51 | 17.4±3.1 | 16.0±3.33* | 15.9±3.21 | 16.7±1.95 | 16.1±2.02 | 15.5±1.65 |
| Calcium (mg/dl) | 11.0±2.92 | 9.3±0.66 | 10.1±1.84 | 9.5±2.23 | 9.8±2.13 | 9.0±1.48 | 10.2±1.51 | 9.7±2.27 |
| Creatine kinase (U/l) | 1538.1±294.5 | 1814.6±456.8 | 1701.9±353.6 | 1587.0±452.3 | 1290.7±175.3 | 1358.9±127.7 | 1436.4±286.9 | 1257.3±150.6 |
| Creatinine (mg/dl) | 0.6±0.11 | 0.6±0.08 | 0.5±0.11 | 0.5±0.1* | 0.4±0.03 | 0.4±0.06 | 0.4±0.1 | 0.4±0.03 |
| GGT (U/l) | 0.7±0.17 | 0.7±0.29 | 0.6±0.3 | 0.6±0.31 | 0.2±0.08 | 0.2±0.07 | 0.2±0.07 | 0.2±0.07 |
| Phosphorous (mg/dl) | 6.6±1.24 | 7.3±0.87 | 7.0±1.18 | 6.1±0.83 | 7.3±0.64 | 7.8±0.97 | 7.5±1.01 | 7.9±0.96 |
| LDH (U/l) | 2673.7±699.7 | 2427.5±745.5 | 1851.2±408.6** | 1862.7±452.3** | 2083.8±178.5 | 2200.4±234.3 | 1821.3±358.7 | 1980.0±422.8 |
| T-Bilirubin (mg/dl) | 0.2±0.09 | 0.2±0.07 | 0.2±0.1 | 0.2±0.09 | 0.2±0.12 | 0.3±0.24 | 0.3±0.23 | 0.2±0.21 |
| TG (mg/dl) | 86.5±22.3 | 75.8±18.1 | 71.5±17.6 | 62.2±22.2 | 68.2±10.0 | 73.4±8.9 | 72.0±10.6 | 71.1±10.1 |
| A/G ratio | 1.6±0.45 | 1.7±0.36 | 1.9±0.48 | 1.4±0.33 | 1.4±0.24 | 1.4±0.13 | 1.3±0.3 | 1.4±0.41 |
| Na (mmol/l) | 115.0±22.75 | 124.3±14.79 | 117.9±14.87 | 104.4±19.33 | 132.3±22.02 | 131.4±18.75 | 144.5±21.47 | 124.6±16.41 |
| K (mmol/l) | 4.3±0.95 | 4.3±0.86 | 4.6±0.61 | 4.0±0.65 | 4.4±0.72 | 4.5±0.69 | 5.4±1.28* | 4.9±1.71 |
| Cl (mmol/l) | 83.7±15.54 | 90.9±10.95 | 87.3±10.11 | 78.8±13.02 | 102.0±16.3 | 99.4±13.88 | 110.0±12.3 | 105.3±20.16 |

Values were expressed as mean±S.D. (n=10).
Significantly different from control at * $p<0.05$ or ** $p<0.01$.
HTNV : Hantaan virus; PUUV : Puumala virus.

**Table 5.** Absolute and relative organ weights in the rats injected subcutaneously with the HTNV-PUUV combination vaccine for 4 weeks

| Sex | Dose (dose/kg) | Absolute (g) | | | | Relative (%) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Organs (g) | 0 | 0.017 | 0.17 | 1.7 | 0 | 0.017 | 0.17 | 1.7 |
| Male | Final body weights | 323.9±14.11 | 331.3±26.72 | 324.2±20.86 | 322.9±23.91 | | | | |
| | Testis (L) | 1.68±0.124 | 1.62±0.063 | 1.66±0.124 | 1.66±0.118 | 0.52±0.02 | 0.49±0.043 | 0.52±0.05 | 0.52±0.058 |
| | Testis (R) | 1.69±0.12 | 1.64±0.101 | 1.65±0.152 | 1.66±0.088 | 0.52±0.02 | 0.5±0.05 | 0.51±0.057 | 0.52±0.048 |
| | Epididymis (L) | 0.44±0.051 | 0.44±0.019 | 0.44±0.036 | 0.46±0.056 | 0.14±0.016 | 0.13±0.012 | 0.14±0.016 | 0.14±0.022 |
| | Epididymis (R) | 0.46±0.038 | 0.44±0.023 | 0.45±0.037 | 0.44±0.038 | 0.14±0.011 | 0.13±0.013 | 0.14±0.015 | 0.14±0.017 |
| | Prostate gland | 0.5±0.143 | 0.41±0.236 | 0.5±0.165 | 0.42±0.142 | 0.16±0.046 | 0.12±0.068 | 0.15±0.043 | 0.13±0.045 |
| | Hypophysis | 0.01±0.002 | 0.01±0.002 | 0.01±0.002 | 0.09±0.001 | 0.003±0.001 | 0.003±0.001 | 0.003±0.001 | 0.003±0 |
| | Brain | 2.0±0.214 | 2.08±0.131 | 2.04±0.099 | 2.05±0.072 | 0.62±0.069 | 0.63±0.057 | 0.63±0.049 | 0.64±0.048 |
| | Thymus | 0.61±0.073 | 0.61±0.131 | 0.57±0.053 | 0.58±0.086 | 0.19±0.025 | 0.18±0.035 | 0.18±0.015 | 0.18±0.035 |
| | Heart | 1.09±0.097 | 1.08±0.113 | 1.09±0.112 | 1.2±0.233 | 0.34±0.028 | 0.33±0.035 | 0.34±0.026 | 0.37±0.087 |
| | Lung | 1.38±0.099 | 1.42±0.153 | 1.4±0.124 | 1.48±0.207 | 0.43±0.022 | 0.43±0.046 | 0.43±0.026 | 0.46±0.072 |
| | Liver | 9.5±0.586 | 9.56±1.504 | 9.81±0.961 | 9.01±0.786 | 2.94±0.245 | 2.89±0.405 | 3.02±0.204 | 2.8±0.272 |
| | Spleen | 0.83±0.105 | 0.84±0.12 | 0.78±0.084 | 0.86±0.121 | 0.26±0.03 | 0.25±0.03 | 0.24±0.02 | 0.27±0.039 |
| | Kidney (L) | 1.21±0.069 | 1.19±0.169 | 1.24±0.093 | 1.19±0.095 | 0.38±0.016 | 0.36±0.048 | 0.38±0.021 | 0.37±0.024 |
| | Kidney (R) | 1.25±0.08 | 1.18±0.142 | 1.26±0.101 | 1.2±0.099 | 0.38±0.017 | 0.36±0.046 | 0.39±0.018 | 0.37±0.023 |
| | Adrenal gland (L) | 0.025±0.003 | 0.025±0.005 | 0.023±0.004 | 0.024±0.003 | 0.008±0.001 | 0.008±0.001 | 0.007±0.001 | 0.008±0.001 |
| | Adrenal gland (R) | 0.024±0.004 | 0.024±0.003 | 0.021±0.005 | 0.025±0.004 | 0.008±0.001 | 0.007±0.001 | 0.006±0.002 | 0.008±0.001 |
| | Thyroid gland (L) | 0.01±0.002 | 0.009±0.002 | 0.009±0.004 | 0.01±0.003 | 0.003±0.001 | 0.003±0.001 | 0.003±0.001 | 0.003±0.001 |
| | Thyroid gland (R) | 0.01±0.002 | 0.008±0.001 | 0.008±0.003 | 0.009±0.001 | 0.003±0.001 | 0.003±0* | 0.003±0.001 | 0.003±0 |
| | Submaxillary gland | 0.55±0.064 | 0.57±0.079 | 0.58±0.068 | 0.62±0.064* | 0.17±0.022 | 0.17±0.026 | 0.18±0.022 | 0.19±0.014* |
| Female | Final body weights | 212.2±11.35 | 211.1±7.65 | 213.4±9.65 | 211.2±11.04 | | | | |
| | Ovary (L) | 0.067±0.009 | 0.069±0.011 | 0.07±0.01 | 0.059±0.007* | 0.032±0.005 | 0.033±0.005 | 0.033±0.005 | 0.028±0.003 |
| | Ovary (R) | 0.072±0.008 | 0.07±0.009 | 0.067±0.013 | 0.062±0.015 | 0.034±0.004 | 0.033±0.005 | 0.032±0.006 | 0.029±0.007 |
| | Uterus | 0.59±0.184 | 0.72±0.139 | 0.56±0.217 | 0.5±0.23 | 0.28±0.098 | 0.25±0.069 | 0.27±0.105 | 0.23±0.111 |
| | Hypophysis | 0.011±0.002 | 0.011±0.002 | 0.012±0.001 | 0.011±0.002 | 0.005±0.001 | 0.005±0.001 | 0.006±0.001 | 0.005±0.001 |
| | Brain | 2.03±0.05 | 1.98±0.052* | 1.94±0.053** | 1.9±0.138* | 0.96±0.05 | 0.94±0.048 | 0.92±0.044 | 0.9±0.08 |
| | Thymus | 0.51±0.072 | 0.47±0.083 | 0.5±0.071 | 0.5±0.078 | 0.24±0.034 | 0.23±0.04 | 0.24±0.035 | 0.24±0.033 |
| | Heart | 0.8±0.082 | 0.81±0.054 | 0.81±0.034 | 0.79±0.056 | 0.38±0.036 | 0.38±0.03 | 0.38±0.019 | 0.38±0.032 |
| | Lung | 1.21±0.095 | 1.19±0.094 | 1.17±0.119 | 1.09±0.323 | 0.57±0.047 | 0.57±0.05 | 0.55±0.053 | 0.51±0.149 |
| | Liver | 6.24±0.498 | 6.26±0.573 | 6.03±0.591 | 5.81±0.476 | 2.95±0.287 | 2.97±0.248 | 2.85±0.219 | 2.75±0.151 |
| | Spleen | 0.67±0.04 | 0.65±0.071 | 0.63±0.091 | 0.63±0.053 | 0.32±0.02 | 0.31±0.034 | 0.3±0.041 | 0.3±0.021* |
| | Kidney (L) | 0.80±0.066 | 0.81±0.055 | 0.81±0.048 | 0.8±0.068 | 0.38±0.026 | 0.38±0.026 | 0.39±0.025 | 0.38±0.039 |
| | Kidney (R) | 0.81±0.051 | 0.82±0.05 | 0.81±0.045 | 0.8±0.07 | 0.38±0.022 | 0.39±0.023 | 0.39±0.033 | 0.38±0.036 |
| | Adrenal gland (L) | 0.035±0.005 | 0.034±0.007 | 0.035±0.006 | 0.032±0.004 | 0.017±0.003 | 0.016±0.003 | 0.017±0.003 | 0.015±0.002 |
| | Adrenal gland (R) | 0.033±0.005 | 0.034±0.004 | 0.037±0.017 | 0.032±0.006 | 0.016±0.002 | 0.016±0.002 | 0.017±0.008 | 0.015±0.003 |
| | Thyroid gland (L) | 0.008±0.002 | 0.008±0.002 | 0.006±0.002 | 0.008±0.006 | 0.004±0.001 | 0.004±0.001 | 0.003±0.001 | 0.004±0.002 |
| | Thyroid gland (R) | 0.007±0.002 | 0.008±0.002 | 0.006±0.002 | 0.008±0.002 | 0.004±0.001 | 0.004±0.001 | 0.003±0.001 | 0.004±0.001 |
| | Submaxillary gland | 0.45±0.054 | 0.46±0.051 | 0.45±0.073 | 0.43±0.042 | 0.21±0.028 | 0.22±0.025 | 0.21±0.037 | 0.2±0.018 |

Values were expressed as mean±S.D. (n = 10). Significantly different from control at * $p<0.05$ or ** $p<0.01$. HTNV : Hantaan virus; PUUV : Puumala virus.

**Table 6.** Histopathologic findings in the rats injected subcutaneously with the HTNV-PUUV combination vaccine for 4 weeks

| Organs | Sex | Male | | | | Female | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Dose (dose/kg) | 0 | 0.017 | 0.17 | 1.7 | 0 | 0.017 | 0.17 | 1.7 |
| | No. of animal | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |
| Kidney | | | | | | | | | |
| Cell infiltration in interstitial tissue | | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| Vacuolation of renal tubules | | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 |
| Spleen | | | | | | | | | |
| Hyperplasia of lymphoid follicles | | 1 | 0 | 1 | 3 | 2 | 1 | 1 | 0 |
| Liver | | | | | | | | | |
| Microgranuloma | | 1 | 0 | 1 | 2 | 1 | 0 | 1 | 2 |
| Vacuolation of hepatocytes | | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| Cell infiltration in Glisson's sheath | | 0 | 1 | 0 | 1 | 0 | 0 | 1 | 0 |
| Lung | | | | | | | | | |
| Thickening of alveolar wall | | 1 | 0 | 0 | 1 | 1 | 2 | 1 | 0 |

HTNV : Hantaan virus; PUUV : Puumala virus.

was also observed in 1 male at the 0.017 dose/kg, 1 male at the control and 1 female in the 0.17 dose/kg group. In liver, microgranuloma in sinusoid was observed in 2 males and 2 females at the 1.7 dose/kg, 1 male and 1 female at the 0.17 dose/kg and 1 male and 1 female at the control group, respectively. In addition, cell infiltraion in Glisson's sheath and vacuolization of hepatocytes were observed in each 1 male in the 0.017 and 1.7 dose/kg/group. However, those were only observed in 1 female of the 0.17 dose/kg group. Hyerplasia of lymphoid follicles were observed in 1 male of the control, 1 male of the 0.17 and 3 males of the 1.7 dose/kg group, and 1 female of the 0.017 dose/kg group, 1 female of the 0.17 dose/kg group and 2 females of the 1.7 dose/kg group. No other abnormal lesions were found.

## IV. DISCUSSION

Recently, some combined vaccines are available; the combined live inactivated virus vaccines against measles, mumps and rubella, and against Japanese encephalitis and Hantaan virus. The combined vaccines have many advantages over the corresponding monovalent vaccines in the practical use such as manufacturing costs, storage and administration. Korea Green Cross Co. Ltd. developed a HTNV-PUUV combination vaccine to prevent HFRS from HTNV and PUUV in human. Total amount of protein and myelin basic protein of Hantavax™ and PUUV vaccine are 10 μg/ml and 0.01 ng/ml, respectively. These amounts, cause of toxic effects, are far less than the WHO-required limits. The potential usefuless of the combined vaccine has been confirmed (Lee *et al.*, 1999). Previous study confirmed that Hantavax™ vaccine is safe in humans following a dose schedule of two doses within one month. The general and local side effects such as mild itching, induration and swelling at the site of injection were registered, and onset of symptoms after vaccination was mostly within a day (Cho and Howard, 1999). However, there has not been any detailed investigation of safety of an inactivated HTNV-PUUV combination vaccine in preclinical study.

In the present study, repeated dose toxicity of a combined HTNV and PUUV vaccine was investigated in male and female SD rats. Test substance was injected subcutaneously to rats at dose levels of 0, 0.017, 0.17 and 1.7 dose/kg once a day for 4 weeks. This dose level is equal to 1, 10 and 100 times of a dosage used clinically in human. No mortality was observed in rats injected with the combined vaccine in the study. During the observation period, there were no abnormal clinical signs related to the combined vaccine in animals. The body weights of rats injected with the vaccine were not statistically significant differences. In addition, food consumption and water intake did not appeare to significant changes following injection of the combined vaccine. Even though some items in hematologic and blood chemical parameters were shown significant differences in animals treated with high dose of the combined vaccine, these parameters were mostly in the normal

range physiologically. These parameters were not revealed evidence of specific toxicity related to the combined vaccine following dose-dependent injection. At autopsy, all organs were examined for gross findings and histopathology. However, the combined vaccine did not induce any abnormal changes at the doses used in the study.

In conclusion, the HTNV-PUUV combination vaccine against HTNV and PUUV may have no signs of the repeated dose toxicity and it is considered to have no toxic effects in the SD rats under the current study condition.

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