Korean J. Toxicol.
Vol. 13, No. 3, pp. 281-291, 1997

Subacute Toxicity of Combined Vaccine (KGCC-95VI) Against Japanese Encephalitis and Hantaan Virus Infection in Rabbits

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(Received May 29, 1997)
(Accepted September 2, 1997)

ABSTRACT *: The subacute toxicity of the combined vaccine (KGCC-95VI) for the prophylaxis against Japanese encephalitis and Hantaan virus infection, recently developed by Korea Green Cross Corporation, was investigated. KGCC-95VI was subcutaneously administered into the both sexes of New Zealand White rabbits at the dosage of 0, 10, 50 and 250 ml/kg body weight (20, 100 and 500 times the expected clincal dose) once a day for 30 days. There were no deaths and clinical findings during the experiment period. In both sexes, there were no statistically significant differences between the treated and control groups in urinalysis tests, hematological tests, blood chemistry tests and pathological examinations. The KGCC-95VI is considered not to have the subacute toxicity in the rabbits.*

Key Words *: Japanese encephalitis, Hantaan virus infection, Vaccine, Subacute toxicity, Rabbits*

I. INTRODUCTION

Japanese encephalitis virus (JEV) is widely distributed in Asia, including Korea, Japan, China, Taiwan, Philippines, far-eastern Russia, all of Southeast Asia and India (Hoke *et al.*, 1988). Mitamura *et al.*, (1938) isolated the virus from the mosquito, *Culex tritaeniorhynchus*. It is established that pigs and birds are the principal viremic hosts and that *Culex tritaeniorhynchus* is responsible for transmission between these vertebrates and from them to humans (Buescher and Scherer, 1959). To date, the inactivated vaccine using the virus propagated in the brains of the suckling mice is being used (Cho *et al.*, 1994).

Hantaan virus was originally isolated from the Korean striped field mouse, *Apodemus agarius* corea. The virus is one of the etiologic agents of hemorrhagic fever with renal syndrome (Hantaan virus infection, Leptospirosis, Rickettial infection). To date, the inactivated vaccine using the virus propagated in the brains of the newborn mice is being used (Shin, 1992; Lee and Ahn, 1988).

Recently Korea Green Cross Corporation developed, for the convenience in practical immunization, the combined vaccine for the prophylaxis against Japanese encephalitis and Hantaan virus infection. The efficacy of the combined vaccine was confirmed. In this study the subacute toxicity of the combined vaccine was investigated using New Zealand White rabbits following the guidelines on the safety tests of the drugs (Guidelines for Safety Tests of Drugs, 1996) provided by the Food and Drug Administration, Korea.

II. MATERIALS AMD METHODS

The test material, combined inactivated virus vaccine for Japanese encephalitis and hantaan virus-caused hemorrhagic fever with renal syndrome (re-

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ferred to as KGCC-95VI hereinafter for convenience), was produced and supplied by Korea Green Cross Corporation based in Korea. Phosphate buffered-saline, 1/60 M, pH 7.2, prepared and autoclaved at the laboratory was used as the diluent for the test material.

Both sexes (12 rabbits of each sex) of New Zealand White rabbits (Laboratory of Experimental Animals, Korea) were obtained at the age of 3 months. All rabbits were acclimatized for 1 week prior to the administration of the test material under the barrier-sustained animal room maintained at a temperature of 23±3°C, a relative humidity of 50± 10% and illumination cycle of 12 hr light and 12 hr dark (light during 07:00-19:00). The rabbits were housed in the automatic washing cages (Dae-Jong, Korea) and fed with new-born calf pellets (Jeil Feed Co., Korea) and tap water *ad libitum*. Each sex group was divided into four subgroups according to the dosage levels.

Following the guidelines on the safety tests of the drugs provided by the Food and Drug Administration, Korea, the possibility of subacute toxicity of the KGCC-VI was investigated. Groups of 3 male and 3 female rabbits received subcutaneous doses of 0 (control), 10, 50 and 250 ml/kg body weight, once a day for 30 days. The animals were inspected daily for the clinical signs. Body weights, food consumption and water consumption were determined twice a week during the administration period. Urine samples were collected and analyzed for specific gravity, pH, protein, ketone bodies, occult blood, bilirubin, urobilinogen, nitrite, leucocytes and glucose using the test strip (Combur-10 Test M, Boehringer Mannheim), and urine volume for 24 hours at the last week of the study. All animals were anesthetized with ether, bled for hematology and blood biochemistry through the posterior vena cava, and necropsided at the termination of the study.

In hematology, leucocyte count, erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpscular hemoglobin concentration and platelet count were determined by a hematological autoanalyzer (S-880 Coulter Counter, Coulter Electronics). Differential leucocyte count was determined on the blood smears. Prothrombin time of plasma was determined by fibro-coagulation analyzer (COBAS).

Blood biochemistry was carried out to determine the serum levels of sodium, aspartate aminotransferase, alanine aminotransferase, total cholesterol, glucose, total bilirubin total protein, triglycerides, alkaline phosphatase, chloride, creatinine, blood urea nitrogen, potassium and albumin by biochemical autoanalyzer (RA-XT, Technicon, USA). Organ weights were measured for kidney, liver, spleen, heart, lung, thyroid, adrenal gland, brain, testis and ovary. These tissues for organ weights, streunum, skin and grossly abnormal tissue were taken and fixed in 10% neutral buffered formalin. The tissues were processed for standard paraffin embedding prior to sectioning at 5 μm and stainded with hematoxylin and eosin. They were examined under the light microscope.

III. RESULTS

1. Clinical Findings

There were no death in all the groups throughout the experiment period. There were no distinguishable clinical signs in the treated groups comparing to the control groups. Morever even though the animals were administered once a day for 30 days consecutively, there were no lesions on the skin.

2. Body Weights, Feed Consumption and Water Consumption

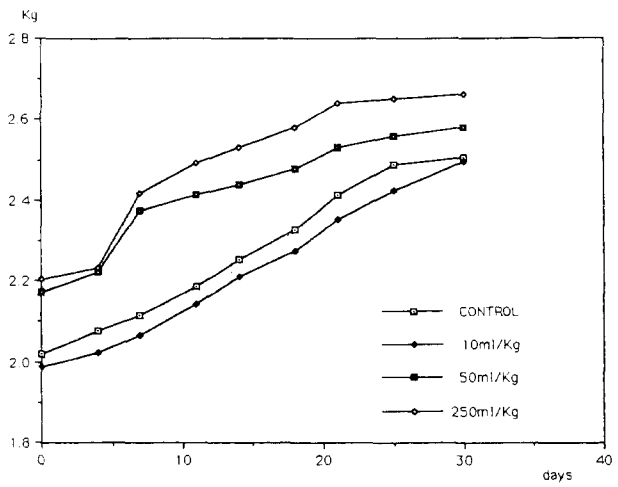

Fig. 1. Body weight changes of male rabbits administered subcutaneously with KGCC-VI once a day for 30 days.

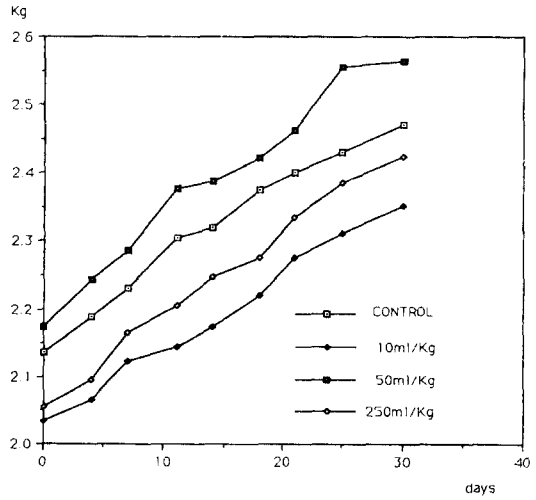

Fig. 2. Body weight changes of female rabbits administered subcutaneously with KGCC-VI once a day for 30 days.

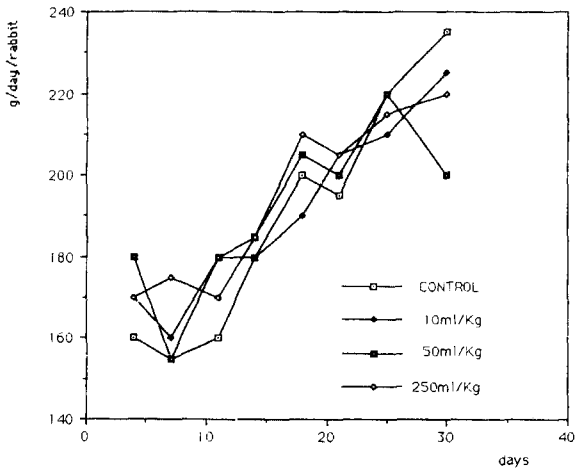

Fig. 3. Food consumption of male rabbits administered subcutaneously with KGCC-VI once a day for 30 days.

There were no statistically significant changes in the body weight, food consumption and water consumption between the treated groups and the control groups (Figs 1, 2 and 3).

3. Urinalysis

In both male and female, there were no statistically significant differences in the measurements between the treated groups and the control groups. The summarized results were shown in Table 1 for the males and Table 2 for the females.

4. Hematology

The results were summarized in Table 3 for the males and Table 4 for the females.

5. Blood Biochemistry

The blood analyte values are summarized in Table 5 for the males and Table 6 for the females.

6. Organ Weights

The absolute organ weights in milligram are summarized in Table 7 for the males and Table 9 for the females. The relative organ weights (organ weight/body weight * 100) in % are summarized in Table 8 for the males and Table 10 for the females.

7. Necropsy Findings

There were no gross pathological findings in all the animals.

8. Histopathological Findings

Mild to moderate inflammations were observed

Table 1. Urinalysis data of male rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Groups		Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals		3	3	3	3
Specific gravity		1.010	1.015	1.010	1.010
pH		8.0	8.0	8.0	8.0
Leukocytes	neg.	3	3	3	3
	25 cells/μl	0	0	0	0
	75 cells/μl	0	0	0	0
	500 cells/μl	0	0	0	0
Nitrite	neg.	3	3	3	3
	pos.	0	0	0	0

Table 1. Continued.

Groups		Control	10 ml/kg	50 ml/kg	250 ml/kg
Protein	neg.	2	2	2	2
	0.3 g/l	1	1	1	1
	1.0 g/l	0	0	0	0
	5.0 g/l	0	0	0	0
Glucose	mormal	3	3	3	3
	0.5 g/l	0	0	0	0
	1.5 g/l	0	0	0	0
	3.0 g/l	0	0	0	0
	10.0 g/l	0	0	0	0
Ketone bodies	neg.	3	3	3	3
	+	0	0	0	0
	++	0	0	0	0
	+++	0	0	0	0
urobilinogen	norma	3	3	3	3
	10 mg/l	0	0	0	0
	40 mg/l	0	0	0	0
	80 mg/l	0	0	0	0
	120 mg/l	0	0	0	0
Bilirubin	neg.	3	3	3	3
	+	0	0	0	0
	++	0	0	0	0
	+++	0	0	0	0
Occult blood	neg.	3	3	3	3
	pos.	0	0	0	0
Erythrocyte	neg.	3	3	3	3
	10 cells/μl	0	0	0	0
	50 cells/μl	0	0	0	0
	250 cells/μl	0	0	0	0

Table 2. Urinalysis data of female rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Groups		Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals		3	3	3	3
Specific gravity		1.010	1.015	1.010	1.010
pH		8.0	8.0	8.0	8.0
Leukocytes	neg.	3	3	3	3
	25 cells/μl	0	0	0	0
	75 cells/μl	0	0	0	0
	500 cells/μl	0	0	0	0
Nitrite	neg.	3	3	3	3
	pos.	0	0	0	0
Protein	neg.	2	2	2	2
	0.3 g/l	1	1	1	1
	1.0 g/l	0	0	0	0
	5.0 g/l	0	0	0	0
Glucose	normal	3	3	3	3
	0.5 g/l	0	0	0	0
	1.0 g/l	0	0	0	0
	3.0 g/l	0	0	0	0
	10.0 g/l	0	0	0	0

Table 2. Continued.

Groups		Control	10 ml/kg	50 ml/kg	250 ml/kg
Ketone bodies	neg.	3	3	3	3
	+	0	0	0	0
	++	0	0	0	0
	+++	0	0	0	0
urobilinogen	norma	3	3	3	3
	10 mg/l	0	0	0	0
	40 mg/l	0	0	0	0
	80 mg/l	0	0	0	0
	120 mg/l	0	0	0	0
Bilirubin	neg.	3	3	3	3
	+	0	0	0	0
	++	0	0	0	0
	+++	0	0	0	0
Occult blood	neg.	3	3	3	3
	pos.	0	0	0	0
Erythrocyte	neg.	3	3	3	3
	10 cells/μl	0	0	0	0
	50 cells/μl	0	0	0	0
	250 cells/μl	0	0	0	0

Table 3. Hematology data of male rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Group	Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals	3	3	3	3
WBC				
Total ($\times 10^3$/mm^3)	9.5(0.67)	9.3(0.74)	9.9(1.42)	9.7(1.46)
Pseudoeosinophi (%)	15.0(4.1)	15.3(3.7)	14.5(4.8)	15.2(3.7)
Lymphocyte (%)	80.3(8.3)	79.3(7.3)	81.7(8.7)	79.8(8.2)
Monocyte (%)	1.5(0.9)	2.6(0.8)	1.9(0.7)	2.7(1.2)
Stab cell (%)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)
Eosinophil (%)	1.8(0.8)	1.8(0.3)	1.3(0.8)	0.9(0.2)
Basophil (%)	1.3(0.4)	1.0(0.2)	0.6(0.3)	1.4(0.5)
Reticulocyte (% of RBC)	1.1(0.2)	1.5(0.3)	1.7(0.5)	0.8(0.2)
RBC ($\times 10^6$/mm^3)	5.8(0.4)	5.6(0.7)	6.0(0.5)	5.5(0.6)
Hemoglobin (g/dL)	12.2(0.8)	11.7(1.2)	12.3(0.9)	11.5(0.7)
Hematocrit (%)	39.5(4.7)	38.9(3.6)	40.3(0.9)	36.8(4.2)
MCV (μmm^3)	68.1(1.1)	69.4(5.1)	67.1(5.5)	66.9(1.4)
MCH (pg)	21.0(0.2)	20.8(0.4)	20.5(0.6)	20.9(0.6)
MCHC (g/dL)	30.8(0.7)	30.1(0.5)	30.5(0.4)	31.2(0.7)
PLT ($\times 10^3$/mm^3)	167(37)	237(49)	264(53)	249(35)

The numbers in parentheses denote the standard deviation.

Table 4. Hematology data of female rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Group	Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals	3	3	3	3
WBC				
Total ($\times 10^3$/mm^3)	8.7(0.43)	9.2(0.58)	7.9(1.56)	8.5(1.38)
Pseudoeosinophi (%)	14.3(3.7)	19.6(4.2)	14.9(3.9)	16.4(4.7)
Lymphocyte (%)	82.1(11.5)	76.0(10.3)	80.6(9.5)	79.0(9.5)
Monocyte (%)	1.4(0.5)	1.9(0.5)	2.4(0.6)	2.6(1.0)
Stab cell (%)	0.0(0.0)	0.0(0.0)	0.0(0.0)	0.0(0.0)

Table 4. Continued.

Group	Control	10 ml/kg	50 ml/kg	250 ml/kg
Eosinophil (%)	1.4(0.6)	1.3(0.3)	1.2(0.3)	0.9(0.3)
Basophil (%)	0.8(0.2)	1.2(0.3)	0.9(0.3)	1.1(0.4)
Reticulocyte (% of RBC)	1.2(0.2)	0.8(0.1)	1.9(0.4)	1.5(0.4)
RBC ($\times 10^6$/mm^3)	5.7(0.5)	5.6(0.4)	5.8(0.6)	5.7(0.7)
Hemoglobin (g/dL)	10.8(1.1)	12.4(0.9)	11.3(0.8)	11.7(0.5)
Hematocrit (%)	37.5(4.4)	34.5(4.3)	36.7(2.7)	35.2(4.8)
MCV (μmm^3)	65.7(0.8)	61.6(2.1)	63.2(2.6)	61.7(3.4)
MCH (pg)	18.9(1.2)	221.(1.6)	19.4(2.0)	20.2(2.6)
MCHC (g/dL)	28.8(0.6)	35.9(0.9)	30.7(0.9)	33.2(0.7)
PLT ($\times 10^3$/mm^3)	256(43)	218(52)	327(63)	283(46)

The numbers in parentheses denote the standard deviation.

Table 5. Blood analyse values of male rabbits administered subcutaneouly with KGCC-95VI once a day for 30 days

Group	Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals	3	3	3	3
Sodium (mEq/l)	1442.(1.7)	139.5(2.6)	143.1(6.2)	144.6(3.8)
ALT (U/l)	75.4(13.7)	79.6(8.3)	80.1(10.2)	77.4(11.7)
AST (U/l)	57.3(12.4)	48.3(10.2)	45.3(9.7)	47.2(8.1)
Cholesterol (mg/dl)	52.3(22.4)	43.7(16.4)	42.3(12.5)	55.8(11.7)
Glucose (mg/dl)	54.3(8.6)	65.2(18.2)	57.3(14.9)	51.5(12.7)
Total bilurubin (mg/dl)	0.33(0.06)	0.41(0.03)	0.39(0.09)	0.28(0.05)
Total protein (g/dl)	6.69(0.56)	6.75(0.75)	6.42(0.35)	6.32(0.68)
Trigycerides (mg/dl)	114.6(25.4)	124.0(23.7)	118.0(28.9)	122.3(22.7)
ALP (U/l)	126.3(20.3)	116.5(24.1)	109.0(28.3)	125.3(23.1)
Chloride (mEq/l)	97.0(1.0)	92.3(2.6)	95.8(6.3)	98.5(4.2)
Creatine (mg/dl)	2.06(0.35)	1.86(0.35)	1.87(0.23)	1.27(0.15)
BUN (mg/dl)	14.5(2.1)	13.2(2.9)	11.6(2.0)	14.1(2.7)
Potassium ion (mEq/dl)	25.56(0.61)	27.20(0.60)	26.37(1.62)	24.16(2.64)
Albumin (g/dl)	2.83(0.32)	2.85(0.38)	2.69(0.27)	2.65(0.22)

The numbers in parentheses denote the standard deviation.

Table 6. Blood analyse values of female rabbits administered subcutaneouly with KGCC-95VI once a day for 30 days

Group	Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals	3	3	3	3
Sodium (mEq/l)	140.5(2.5)	143.7(3.1)	141.7(5.9)	142.0(4.5)
ALT (U/l)	78.5(12.9)	77.3(18.7)	69.4(13.7)	84.6(15.2)
AST (U/l)	47.3(11.6)	45.8(13.4)	39.2(7.4)	51.2(11.3)
Cholesterol (mg/dl)	39.3(13.8)	44.8(9.4)	40.7(10.1)	45.2(9.7)
Glucose (mg/dl)	63.5(10.2)	62.8(12.2)	57.9(11.5)	56.1(9.6)
Total bilurubin (mg/dl)	0.34(0.08)	0.37(0.07)	0.43(0.11)	0.59(0.15)
Total protein (g/dl)	6.40(0.56)	6.37(0.47)	6.52(0.65)	6.42(0.55)
Trigycerides (mg/dl)	132.1(21.4)	124.6(15.4)	127.3(28.9)	130.4(16.3)
ALP (U/l)	116.8(22.5)	115.3(16.7)	129.0(28.3)	119.6(14.2)
Chloride (mEq/l)	97.0(1.0)	92.0(3.6)	98.0(8.2)	91.3(5.0)
Creatine (mg/dl)	1.93(1.0)	1.77(0.53)	1.82(0.25)	1.73(0.29)
BUN (mg/dl)	1.93(0.26)	13.2(2.7)	13.1(2.8)	15.2(2.3)
Potassium ion (mEq/dl)	13.5(2.3)	26.5(0.5)	24.9(1.2)	26.2(1.1)
Albumin (g/dl)	25.3(0.6)	2.66(0.35)	2.74(0.27)	2.64(0.23)

The numbers in parentheses denote the standard deviation.

at the injection sites of the animals of both sexes dosed at 50 and 250 ml/kg. Nephrocalcinosis was observed in 1 female dosed at 10 ml/kg and 2 females dosed at 50 ml/kg. Other changes, ectopic thymus of the thyroid, interstitial nephritis, hydronephrosis and edema at the injection sites were seen in a few cases without dose-dependency (Table 11 and 12).

Table 7. Absoulte organ weights (mg) of male rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Group		Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals		3	3	3	3
Brain		8850(610)	8760(727)	8843(903)	9303(621)
Spleen		1200(320)	1370(461)	1433(498)	1690(55)
Testis,	left	1230(80)	1050(425)	1163(542)	1933(612)
	right	1230(110)	1010(427)	1290(731)	1165(542)
Heart		6160(730)	6261(528)	6273(933)	7031(1071)
Liver		69190(12900)	78160(11789)	78586(11789)	74313(11824)
Lung		11568(1320)	11943(1222)	10955(548)	13328(698)
Kidney,	left	8040(180)	8210(121)	8743(914)	8826(854)
	right	8130(330)	8486(390)	8942(492)	8546(818)
Adrenal,	left	96(15)	93(15)	96(11)	85(5)
	right	83(21)	76(15)	86(11)	90(10)
Thymus,	left	123(80)	126(32)	103(32)	123(66)
	right	123(11)	146(5)	130(10)	96(53)
Hypothalamus		23(5)	30(10)	26(9)	33(5)
Epiditymis,	left	303(32)	260(91)	260(91)	423(36)
	right	396(115)	353(166)	423(142)	523(144)

The numbers in parentheses denote the standard deviation.

Table 8. Relative organ weights (%) to body weight of male rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Group		Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals		3	3	3	3
Body weight (g)		2504(395)	2496(243)	2579(147)	2653(210)
Brain		.3534(.0154)	.3539(.0299)	.3428(.0614)	.3506(.0295)
Spleen		.0479(.0081)	.0554(.0019)	.0555(.0033)	.0637(.0029)
Testis,	left	.049(.0202)	.4252(.0174)	.4493(.0368)	.7286(.2971)
	right	.0491(.0278)	.4092(.1759)	.5001(.0497)	.4862(.1484)
Heart		.2460(.0184)	.2494(.0303)	.2432(.0635)	.2650(.0150)
Liver		2.7631(.3265)	3.1656(.4716)	3.0475(.8019)	2.8010(.5630)
Lung		.4962(.03342)	.4785(.05031)	.4248(.03728)	.5024(.03327)
Kidney,	left	.3210(.0455)	.3325(.0498)	.3388(.0621)	.3326(.0406)
	right	.3246(.0835)	.3437(.1604)	.3466(.0335)	.3212(.0389)
Adrenal,	left	.0038(.0004)	.0037(.0004)	.0037(.0008)	.0032(.0003)
	right	.0034(.0005)	.0031(.0006)	.0033(.0007)	.0033(.0004)
Thymus,	left	.0049(.0002)	.0051(.0002)	.0040(.0002)	.0046(.0003)
	right	.0049(.0003)	.0059(0.0002)	.0050(.0002)	.0046(.0002)
Hypothalamus		.0009(.0001)	.0012(.0004)	.0011(.0002)	.0012(.0002)
Epiditymis,	left	.0119(.0011)	.0105(.0037)	.0100(.0006)	.0159(.0014)
	right	.0158(.0029)	.0413(.0016)	.0136(.0011)	.0197(.0019)

The numbers in parentheses denote the standard deviation.

Table 9. Absoulte organ weights (mg) of female rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Group		Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals		3	3	3	3
Brain		8926(1122)	8338(4059)	8440(3780)	8546(159)
Spleen		923(195)	1570(408)	5266(2309)	1203(586)
Heart		5066(1267)	6076(923)	3793(674)	5603(2240)
Liver		76803(2135)	74976(5595)	60214(971)	87823(2885)
Lung		10753(1744)	10416(1510)	10603(2525)	987(182)
Kidney,	left	7646(1950)	7733(305)	6210(672)	7900(1307)
	right	7000(1397)	7733(376)	6633(251)	7946(1298)
Adrenal,	left	123(51)	86(30)	100(26)	113(11)
	right	123(51)	93(25)	90(35)	93(35)

Table 9. Continued.

Group		Control	10 ml/kg	50 ml/kg	250 ml/kg
Thymus,	left	136(107)	160(87)	113(66)	116(41)
	right	133(5)	140(10)	96(28)	86(11)
Over,	left	146(32)	116(37)	110(32)	190(10)
	right	146(20)	113(28)	110(30)	175(25)
Hypothalamus		23(5)	26(5)	26(1)	26(5)
Uterus		1976(755)	3656(666)	2733(490)	3860(582)

The numbers in parentheses denote the standard deviation.

Table 10. Relative organ weights (%) to body weight of female rabbits administered subcutaneously with KGCC-95VI once a day for 30 days

Group		Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals		3	3	3	3
Body weight(g)		2471(114)	2353(264)	2563(839)	2425(340)
Brain		.3612(.0984)	.3543(.0401)	.3293(.0275)	.3524(.04670)
Spleen		.3735(.0171)	.6672(.0154)	.4054(.0168)	.4960(.0170)
Heart		.2050(.0011)	.2582(.0353)	.2497(.0491)	.2310(.0658)
Liver		3.1080(.1872)	3.1864(.2119)	3.2492(.0708)	3.6214(.4470)
Lung		.4353(.06535)	.4427(.05721)	.4137(.06269)	.4072(.05377)
Kidney,	left	.3094(.0171)	.3286(.0115)	.3422(.0490)	.3257(.0384)
	right	.2832(.0122)	.3286(.0142)	.3587(.0183)	.3276(.0381)
Adrenal,	left	.0039(.0008)	.0039(.0001)	.0039(.0002)	.3038(.0001)
	right	.0039(.0004)	.0036(.0001)	.0039(.0002)	.0038(.0001)
Thymus,	left	.0055(.0001)	.0057(.0003)	.0044(.0004)	.0047(.0001)
	right	.0053(.0003)	.0059(.0003)	.0047(.0002)	.0045(.0003)
Ovary,	left	.0059(.0002)	.0049(.0001)	.0052(.0004)	.0062(.0002)
	right	.0059(.0001)	.0048(.0001)	.0053(.0002)	.0056(.0002)
Hypothalamus		.0009(.0000)	.0011(.0001)	.0010(.0001)	.0010(.0001)
Uterus		.0799(.0015)	.1553(.0636)	.2859(.0357)	.1591(.0105)

Table 11. Histopathological findings of male rabbits administered subcutaneoulsy with KGCC-95VI once a day for 30 days

Group	Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals	3	3	3	3
Liver, focal necrosis	n. f.	n. f.	n. f.	n. f.
lymphocytes increased in Glisson's sheath	n. f.	n. f.	n. f.	n. f.
Kupffer cell hyperplasia	n. f.	n. f.	n. f.	n. f.
mild atrophy of hepatocyte	n. f.	n. f.	n. f.	n. f.
Spleen, hemosiderosis	n. f.	n. f.	n. f.	n. f.
extramedulary hematopoiesis	n. f.	n. f.	n. f.	n. f.
mild increase in granulocytes in red pulp	n. f.	n. f.	n. f.	n. f.
Salivary gland	n. f.	n. f.	n. f.	n. f.
Trachea	n. f.	n. f.	n. f.	n. f.
Esophagus	n. f.	n. f.	n. f.	n. f.
Urinary bladder	n. f.	n. f.	n. f.	n. f.
Heart	n. f.	n. f.	n. f.	n. f.
Ovary	n. f.	n. f.	n. f.	n. f.
Uterus, neutrophil migration in mucosa	n. f.	n. f.	n. f.	n. f.
Epididymis	n. f.	n. f.	n. f.	n. f.
Pancrease	n. f.	n. f.	n. f.	n. f.
Thymus	n. f.	n. f.	n. f.	n. f.
GIT, neutrophil migration in mucosal layer	n. f.	n. f.	n. f.	n. f.
Stomach, lmphocyte aggregation in submucosa	n. f.	n. f.	n. f.	n. f.
Cerebrum, leptomeningits	n. f.	n. f.	n. f.	n. f.
granulomatous immflamation	n. f.	n. f.	n. f.	n. f.
Cerebellum	n. f.	n. f.	n. f.	n. f.
Kidneys, focal interstial nephrities	n. f.	n. f.	n. f.	n. f.
Lungs, peribronchiolar lymphoid cell aggregation	n. f.	n. f.	n. f.	n. f.

n. f. : no findings

Table 12. Histopathological findings of female rabbits administered subcutaneoulsy with KGCC-95VI once a day for 30 days

Group	Control	10 ml/kg	50 ml/kg	250 ml/kg
Number of animals	3	3	3	3
Liver, focal necrosis	n. f.	n. f.	n. f.	n. f.
lymphocytes increased in Glisson's sheath	n. f.	n. f.	n. f.	n. f.
Kupffer cell hyperplasia	n. f.	n. f.	n. f.	n. f.
mild atrophy of hepatocyte	n. f.	n. f.	n. f.	n. f.
Spleen, hemosiderosis	n. f.	n. f.	n. f.	n. f.
extramedulary hematopoiesis	n. f.	n. f.	n. f.	n. f.
mild increase in granulocytes in red pulp	n. f.	n. f.	n. f.	n. f.
Salivary gland	n. f.	n. f.	n. f.	n. f.
Trachea	n. f.	n. f.	n. f.	n. f.
Esophagus	n. f.	n. f.	n. f.	n. f.
Urinary bladder	n. f.	n. f.	n. f.	n. f.
Heart	n. f.	n. f.	n. f.	n. f.
Ovary	n. f.	n. f.	n. f.	n. f.
Uterus, neutrophil migration in mucosa	n. f.	n. f.	n. f.	n. f.
Epididymis	n. f.	n. f.	n. f.	n. f.
Pancrease	n. f.	n. f.	n. f.	n. f.
Thymus	n. f.	n. f.	n. f.	n. f.
GIT, neutrophil migration in mucosal layer	n. f.	n. f.	n. f.	n. f.
Stomach, lmphocyte aggregation in submucosa	n. f.	n. f.	n. f.	n. f.
Cerebrum, leptomeningits	n. f.	n. f.	n. f.	n. f.
granulomatous immflamation	n. f.	n. f.	n. f.	n. f.
Cerebellum	n. f.	n. f.	n. f.	n. f.
Kidneys, focal interstial nephrities	n. f.	n. f.	n. f.	n. f.
Lungs, peribronchiolar lymphoid cell aggregation	n. f.	n. f.	n. f.	n. f.

n. f. : no findings

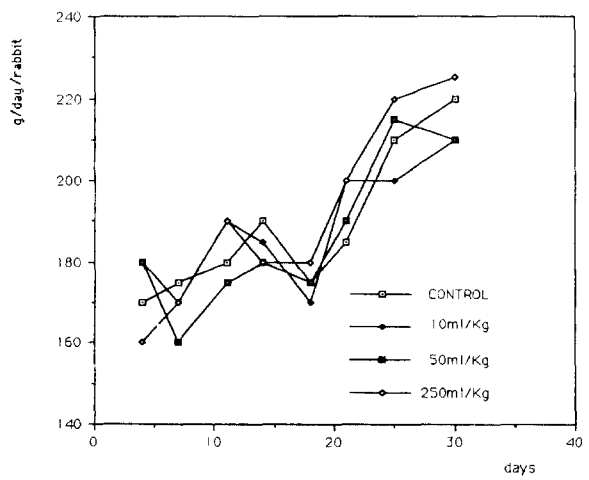

Fig. 4. Food consumption of female rabbits administered subcutaneously with KGCC-VI once a day for 30 days.

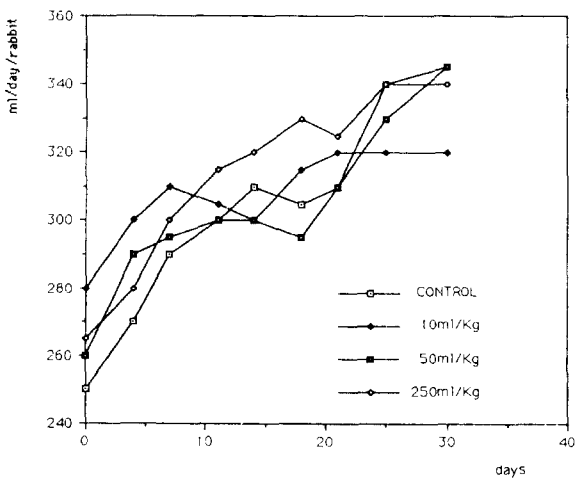

Fig. 5. Water consumption of male rabbits administered subcutaneously with KGCC-VI once a day for 30 days.

IV. DISCUSSION

The practical use of Japanese encephalitis vaccine, purified from infected mouse brains started in 1966 in Japan, has led to a rapid increase in the number of the vaccinated people and a rapid reduction in the incidence of this disease (Oya, 1987). At the beginning of the use of the vaccine, the virus was purified by alcohol-protamine precipitation and centrifugation. The vaccine contained a high level of impurities and its potency was very low. Recently the manufacturing procedures employs many sophisticate methods such as ultracentrifugation and ultrafiltration to reduce the impurities and improve the potency (Umenai *et al.*, 1985).

In 1988, Lee and Ahn and Yamanishi *et al.* reported the development of inactivated vaccines against HFRS with Hantaan virus infection. Lee

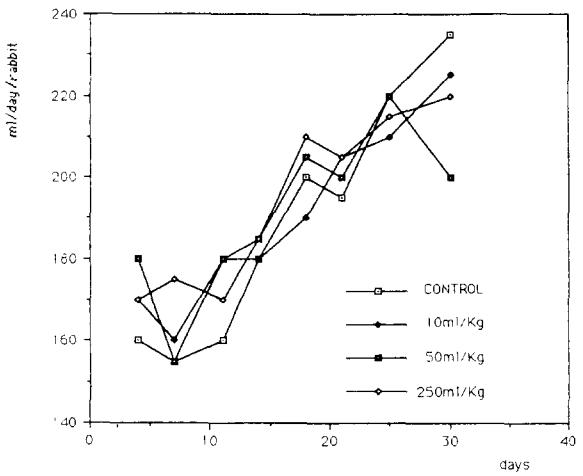

Fig. 6. Water consumption of female rabbits administered subcutaneously with KGCC-VI once a day for 30 days.

and Ahn inoculated Hantaan virus isolated from an HFRS patient into the suckling rat brains and purified and inactivated with the methods to prepare Japanese encephalitis virus mouse brain vaccine with a slight modification. Yamanishi *et al.*, (1988) inoculated Seoul virus isolated from a rat tumor into the suckling mouse brains. The available evidences appeared that these vaccines induced protective immunity in mice.

Currently some combined vaccines are available. The toxoid vaccines against tetanus and diphtheria and inactivated pertussis whole cell vaccine were combined. The live attenuated virus vaccine against measles, mumps and rubella were combined. The combined vaccines have many advantages over the corresponding monovalent vaccines in the practical use such as manufacturing costs, transportation, storage and administration. The combined vaccines may result in a substantially reduced number of contacts with health care workers to immunize against those diseases. The cost of administration a vaccine is at least 10 times the cost of the vaccine (Douglas, 1993). Although it is unlikely that this ratio will hold for many other vaccines, reducing the number of visits of health care workers for vaccine administration could clearly result in great savings.

In this study, the subacute toxicity of the combined vaccine against Japanese encephalitis and Hantaan virus infection (KGCC-95VI) was investigated using the New Zealand White rabbits. The KGCC-95VI was administered to the New Zealand White rabbits with 20, 100 and 500 times the expected clinical dose (0.5 m*l* dose) subcutaneously once a day for 30 days. There were no deaths or clinical signs that might be related to the KGCC-95VI. In both male and female, there were no statistically significant differences between the treated groups and the control groups in the body weight changes, feed and water consumption, urinalysis, hematological tests, blood chemistrical tests and pathological observations.

In 1995 Lim *et al.* administered the HRccine (Hantaan virus vaccine) into the Japanese White rabbits with 300, 60 and 12 times the expected clinical dose and concluded that the subacute toxicity of the vaccine is more than 300 times the human dose. The HRccine and the KGCC-95VI are not biologically identical. However, in this study administration of 500 times the expected clinical dose did not show any abnormalities in all the tests.

In conclusion, the combined vaccine (KGCC-95VI) against Japanese encephalitis and Hantaan virus infection showed no signs of the subacute toxicity and is considered not to have the subacute toxic effects in New Zealand White rabbits.

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