



A 6-Week Oral Toxicity Study of Oral Cholera Vaccine in Sprague-Dawley Rats

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The present study was carried out to examine the toxicity and target organs of oral cholera vaccine (OCV) after repeated oral administration in Sprague-Dawley rats for 6 weeks (3 administrations, once every 2 weeks). OCV is an inactivated oral cholera vaccine that contains *Vibrio cholerae* and confers protection against cholera caused by *V. cholera* serogroups O1 (Inaba and Ogawa serotypes) and O139 (strain 4260B). The animals were orally administered either OCV placebo (negative control) or OCV at a dose equivalent to 240 times the anticipated human dose. Throughout the administration period, no significant change was detected in clinical signs, body weight, food or water consumption, urinalysis results, hematological and clinical biochemistry test results, organ weights, necropsy, or histopathological examination results. Minor changes were found in hematological and clinical biochemistry tests; however, these changes were within normal ranges. The above results suggest that oral administration of OCV in rats did not induce any toxicologically meaningful changes, and the target organs could not be determined. This study was conducted in accordance with the guidelines established by Good Laboratory Practice (2009-183, KFDA, December 22, 2009) and the OECD Principles of Good Laboratory Practice (1997).

Key words: Oral cholera vaccine, Oral toxicity, Sprague-Dawley rats

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Abbreviations: A/G: Albumin/Globulin ratio, ALB: Albumin, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, APTT: Activated partial thromboplastin time, AST: Aspartate aminotransferase, BASO: Basophils, BIL: Bilirubin, BUN: Blood urea nitrogen, CPK: Creatine phosphokinase, CRE: Creatinine, EOS: Eosinophil, GLU: Glucose, HCT: Hematocrit, HDL: High density lipoprotein, HDW: Hemoglobin distribution width, HGB: Hemoglobin, IP: Inorganic phosphorus, K⁺: Potassium, KET: Ketone body, LUC: Large unstained cell, LYM: Lymphocyte, SD: Standard Deviation, OECD: Organization for Economic Co-operation and Development, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume, MONO: Monocyte, MPV: Mean platelet volume, NEU: Neutrophil, NIT: Nitrite, OB: Occult blood, PLT: Platelet count, PRO: Protein, PT: Prothrombin time, RBC: Red blood cell, RDW: Red cell distribution width, RET: Reticulocyte, SG: Specific gravity, TBIL: Total bilirubin, TCHO: Total cholesterol, TG: Triglyceride, TP: Total protein, URO: Urobilinogen, WBC: White blood cell, SPSS: Statistical Package for the Social Sciences, CV: Coefficient of Variation, KFDA: Korea Food and Drug Administration

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INTRODUCTION

Cholera is an acute dehydrating diarrhoeal disease caused by infection of *Vibrio cholerae* O1 and O139, a Gram-negative bacillus. After an incubation period of 1~3 day, infectee suffers from voluminous diarrhea, some abdominal pain and fever. Besides, acute dehydration usually accompanies hypokalemia, metabolic acidosis and shock. Without treatment, coma, seizures and death can occur (Deen *et al.*, 2008). Cholera is transmitted via the faecal-oral route or intake of water and food contaminated by *V. cholerae*. Because of these reasons, cholera breaks out often after war, civil unrest and natural disasters and remains a main public health problem in developing countries (Zuckerman *et al.*, 2007). The World Health Organization (WHO) has reported 98% of all cholera cases occurred in 45 countries located East Africa corridor, large estuarine deltas in Asia (Ganges, Mekong) and Northwest Africa (World Health Organization, 2010). Cholera is both an epidemic and endemic infectious disease. Epidemic cholera occurs unpredictably and target to at-risk people having little or no background immunity. Therefore, in the outbreak of humanitarian emergencies, children and adult are affected equally (Harris *et al.*, 2010). The recent epidemic of cholera in Haiti responsi-

ble for 452,189 cases and 6,334 deaths in just 11 months, provide a good example (Frerichs *et al.*, 2012). In comparison, endemic cholera have recurrent pattern about time and space. Endemic cholera also affects children and adult. But, because adult have had immunity about endemic cholera more than children, children show severe clinical symptoms and high incidence rate. In endemic regions (Africa, Asia, South America, Central America), cholera occurs 3~5 million case and 100,000~130,000 deaths per year (Zuckerman *et al.*, 2007; Reiner *et al.*, 2012).

Key points of cholera prevention are provision of clean water, adequate sanitation. But in endemic regions, improvements of water and sanitation need to long-term, sustained investments and commitments from governments and donor communities (Lucas *et al.*, 2007). Because of these problems, vaccines are chosen as an immediate alternative for prevention of cholera.

Several types of cholera vaccine already were developed. However, among of them, the injectable cholera vaccine has not used as public health tools due to heavy side effects, short protection period, low efficacy and need for frequent boosters (Lopez *et al.*, 2008). Recently, Oral cholera vaccine (OCV) has been recommended by the WHO. In the WHO's report, use of oral cholera vaccines in certain endemic and epidemic situation can be complementary method about existing strategies for cholera control (World Health Organization, 2004). And there are currently two international licensed OCVs, Dukoral™ (Crucell) and Orochol™ (Crucell). These vaccines have been proven safety and efficacy than the injectable cholera vaccine (Tacket *et al.*, 1999; Trach *et al.*, 1997). However, the amount of these vaccines used was little in endemic and epidemic cholera regions, because they have been used generally for travelers (Clemens, 2011).

To improve these problems, new OCV should be low-cost, high efficacy and convenience of global use. The objective of the present study, therefore, are to estimate the toxicity and to identify the target organ after repeated oral administration of new OCV in Sprague-Dawley rats for 6 weeks (3 times, once every 2 weeks) and further to clarify its safety for clinical use.

This study was conducted in accordance with the Good Laboratory Practice for Nonclinical Studies (2009-183, KFDA, Dec 22, 2009) and the OECD Principles of Good Laboratory Practice (1997).

MATERIALS AND METHODS

Animal and husbandry. Male and female Sprague-Dawley rats (5-week old upon receipt, Koatech Co. Ltd, Korea) were used after acclimatization for 7 days. Animals were allocated two per stainless steel cage (W 215 × L 355 × H 200 mm). Environmental controls were set to maintain following conditions: temperature range of 23 ± 3°C, rela-

tive humidity range of 55 ± 15%, ventilation of 10~20 air changes/hr, 150~300 Lux of luminous intensity and a 12-hr light/12-hr dark cycle. Animals were offered irradiation-sterilized pellet diet for lab animal (Teklad certified irradiated global 18 % protein rodent diet, 2918C, Harlan Laboratories Inc., USA) purchased from from Koatech Co., Ltd. (406 Dongcheon-ri, Jinwi-myeon, Pyeongtaek-si, Gyeonggi-do, Korea), *ad libitum*. Groundwater disinfected by ultraviolet sterilizer and ultrafiltration were given via water bottle, *ad libitum*. Analysis of water was performed by an authorized Gyeonggi-do Institute of Health & Environment (324-1, Pajang-dong, Jangan-gu, Suwon-si, Gyeonggi-do, Korea), and there were no factors that could affect results. All animals were over night fasted before dosing and terminal necropsy. Animals were individually distinguished by fur marking method and ear punch (acclimatization period: tail marking method). Individual cages were distinguished by color-coded ID cards, and cage racks were given unique serial numbers. A log sheet about animal use was attached at the entrance of the animal room.

Test article and dosage. The test article OCV, supplied by the Eubiologics, is a yellow to yellowish suspension and its main components are total 5 types of inactivated V. cholera O1 and O139 strains: (1) heat killed Inaba Cairo 48, (2) formaldehyde killed Inaba Phil 6973 El Tor, (3) heat killed Ogawa Cairo 50, and (4) formaldehyde killed Ogawa Cairo 50 of serogroup O1 and (5) formaldehyde killed 4260B of serogroup O139. And their contents were 1157 L.E.U/1.5 ml of O1 Inaba, 1233 L.E.U/1.5 ml of O1 Ogawa and 842 L.E.U/1.5 ml of O139 4260B. The negative control article, OCV placebo, was also supplied by the Eubiologics.

The production of components is divided into two methods according to how to inactivate V. cholera. In case of heat inactivation, the components are produced thorough the follow process: seed culture, main culture, concentration, washing and heat inactivation. In formaldehyde inactivation, the production processes are as follows: seed culture, main culture, formaldehyde inactivation, concentration, and washing.

The dose of test article was set at 1.5 ml/rat (≅ 6 ml/kg, based on 250 g rat) that was about 240 times the anticipated human dose (1.5 ml/human ≅ 0.025 ml/kg, based on 60 kg adult human) and negative control group was set.

Grouping and dosing. Healthy animals selected after the acclimation period were weighed and randomly assigned to groups.

Animals of negative control group (G1) were dosed with the OCV placebo. Animals of treatment group (G2) were dosed with OCV, about 240 times the anticipated human dose. Oral administration was selected as the intended clinical route. The articles were administered 3 times in total, once every 2 weeks. The dosing was completed no later than 11:30. The prepared test article was

directly injected into stomach using an oral gavage with syringe tube.

Observations of clinical signs. During the observation periods, every animal was daily observed for clinical signs and mortality and recorded individually. After the dosing, clinical signs and mortality were checked continuously for the first one hour focusing especially on the respiration behavior. The first day of dosing was designated as day 0.

Body weights. Animals were weighed on the first day of dosing and once a week thereafter, on the days before and of necropsy. The terminal body weight was measured after fasting overnight.

Food and water consumption. Food and water consumption were measured at the start of dosing and once a week thereafter, and daily food and water consumption (g/head/day) were calculated.

Urinalysis. In the last week 5 animals were housed per group in a metabolic cage for urine collection, and 1 mL of urine sample freshly collected for 3~4 hours was used for urinalysis. Urine collected over 24 hours was used to measure the total volume of urine. Urine color, clarity, GLU, BIL, KET, SG, pH, PRO, URO, NIT and OB were analyzed using the Multistix (10SG, SIEMENS, USA) and an automatic analyzer (CliniTek 100, SIEMENS, USA). The urine color was observed with naked eyes. Urine sediments were stained according to the Sternheimer-Malbin method and WBC, epithelial cells, RBC and casts were examined by a microscope.

Hematological and clinical biochemistry test. Animals for the scheduled necropsy were fasted overnight and anesthetized with inhalation of isoflurane (Ifran liquid Hana Pharm. Co., Ltd.). After anesthesia was confirmed, blood was taken from the posterior vena cava for the hematological and serum biochemical tests. RBC, HGB, HCT, MCV, MCH, MCHC, RDW, HDW, RET, PLT, MPV, WBC, NEU, LYM, MONO, EOS, BASO, LUC, PT and APTT were analyzed with a coulter counter (ADVIA 2120, SIEMENS, USA).

Necropsy. After blood collection, animals were sacrificed by bloodletting from abdominal aorta and posterior vena cava. All the organs of body surface, subcutis, head and all the internal organs of the abdominal & thoracic cavities were observed grossly and recorded. All extracted organs were weighed and fixed.

Organ weights. The organs were weighed (paired organs were weighed separately) with an electronic balance (BP221S, Sartorius AG, Germany), and the organ weights were converted to relative organ weights based on the

organ-to-fasted body weight ratio.

Histopathological examination. All fixed organs were examined. More detailed examination was performed on immune organs.

Statistical analysis. All numerical data were analyzed by the Student's t-test to compare the data from the treatment group with those of the negative control group. The urinalysis data were ranked and compared using a non-parametric method (Kruskal-Wallis' H-test). The commercial statistical program, SPSS 10.1K software, was used for all statistical analyses. Significance was judged at a probability value of $p < 0.05$.

RESULTS

Clinical signs and change of body weight. No mortality or abnormal clinical signs were noted during the study. And the mean body weight gain and fasted body weight at necropsy in the male treatment group were significantly lower ($p < 0.05$) than those in negative control group. There were no significant differences between the treatment group and negative control group in females (Fig. 1).

Food and water consumption. The mean food and water consumption on week 4 in the female treatment group was significantly lower ($p < 0.05$) than that in negative control group. There were no significant differences between

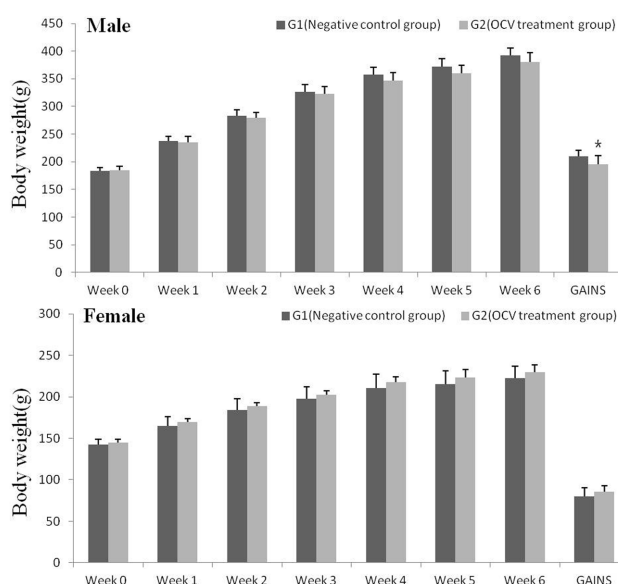


Fig. 1. Body weight increases of male and female SD rats, repeated oral administrations of Oral Cholera Vaccine (OCV) in Sprague-Dawley rats for 6 weeks (3 times, once every 2 weeks). GAIN is body weight on week 6 - body weight on week 0. *Significantly different from the untreated controls ($p < 0.05$).

the treatment group and negative control group in females (Fig. 2 and 3).

Urinalysis. There were no statistically significant differences between the treatment group and negative control

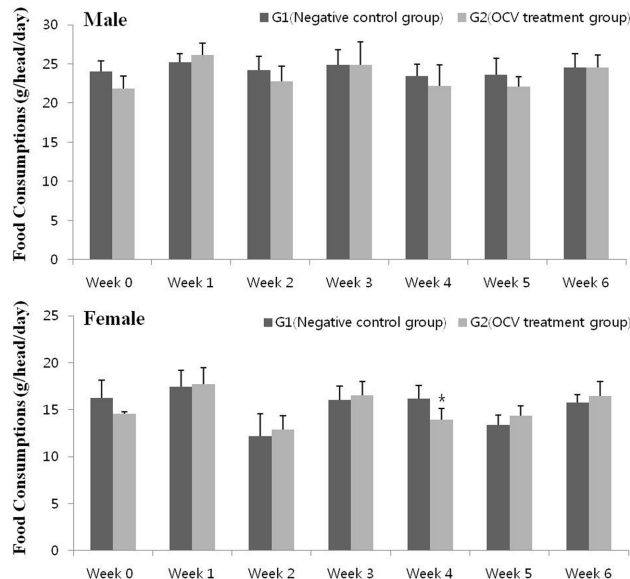


Fig. 2. Food consumption of male and female SD rats, repeated oral administrations of Oral Cholera Vaccine (OCV) in Sprague-Dawley rats for 6 weeks (3 times, once every 2 weeks). *Significantly different from the untreated controls ($p < 0.05$).

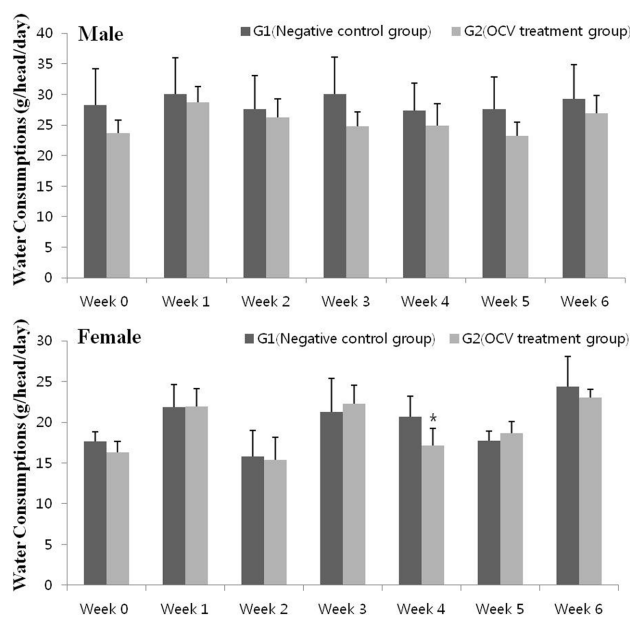


Fig. 3. Water consumption of male and female SD rats, repeated oral administrations of Oral Cholera Vaccine (OCV) in Sprague-Dawley rats for 6 weeks (3 times, once every 2 weeks). *Significantly different from the untreated controls ($p < 0.05$).

group in both sexes (Table 1 and 2).

Hematological and clinical biochemistry test. Hemoglobin concentration (HGB) and Hematocrit (HCT) in male treatment group were significantly higher ($p < 0.01$) than those in the negative control group. There were no significant differences between the treatment group and negative control group in females (Table 3).

Creatinine (CRE) in female treatment group was significantly lower ($p < 0.05$) than that in the negative control group. There were no significant differences between the treatment group and negative control group in males (Table 4).

Table 1. Urinalysis data of OCV treated group and negative control group at the end of administration period

Tests	Result	Groups (dose/head/day)			
		Male		Female	
		G1 (0)	G2 ($\times 240^{ab}$)	G1 (0)	G2 ($\times 240^{ab}$)
GLU	Negative	5	5	5	5
BIL	Negative	5	5	5	5
KET (mg/dl)	Negative	1	0	5	5
	Trace	3	4	0	0
	30	1	1	0	0
	1.005	0	0	1	0
	1.010	0	1	3	3
SG	1.015	0	0	0	2
	1.020	3	2	1	0
	1.025	2	2	0	0
	pH	7.5	1	0	0
PRO (mg/dl)	8.0	3	3	3	2
	8.5	1	2	1	2
	≥ 9.0	0	0	1	0
	Negative	0	0	2	2
	Trace	0	0	1	0
URO (EU/dl)	30	1	1	1	0
	100	4	2	0	3
	≥ 300	0	2	1	0
	0.1	5	5	5	5
NIT	Negative	5	5	5	5
OB	Negative	5	4	4	5
	Small	0	1	0	0
	Large	0	0	1	0
Volume (mL)		17.0 ± 4.6	12.4 ± 3.4	17.2 ± 2.2	13.4 ± 4.0
CLARITY-Clear		5	5	5	5
COLOR-Yellow		5	5	5	5

^{a)} \times AHD (anticipated human dose).

GLU: Glucose, BIL: Bilirubin, KET: Ketone body, SG: Specific gravity, PRO: Protein, URO: Urobilinogen, NIT: Nitrite, OB: Occult blood.

Table 2. Urine sediments of OCV treated group and negative control group at the end of administration period

Tests	Result	Groups (dose/head/day)			
		Male		Female	
		G1 (0)	G2 (× 240 ^a)	G1 (0)	G2 (× 240 ^a)
RBC	0	5	4	4	5
(mean/field)	≤ 4	0	1	0	0
	5~8	0	0	0	0
	9~30	0	0	0	0
	31 ≤ (local)	0	0	0	0
	All over the fields	0	0	1	0
WBC	0	2	2	4	5
(mean/field)	≤ 5	3	3	0	0
	6~20	0	0	1	0
Epithelial cell	0/20 fields	0	1	4	4
	Few/20 fields	2	3	1	0
	Around 1/few fields	2	0	0	1
	Few/field	1	1	0	0
Casts	0	4	4	3	2
(mean/field)	1	1	0	2	3
	2~5	0	1	0	0

^a) ×AHD (anticipated human dose).

Organ weights. The increasing tendency in the absolute and relative weights of the epididymides were observed in male treatment group, and statistical significance ($p < 0.01$) was confirmed in the relative weight of left epididymis and the absolute and relative weights of right one. The relative weight of heart and the absolute weight of liver in the female treatment group were significantly higher ($p < 0.05$) than those in the negative control group (Table 5 and 6).

Necropsy findings. In males, the size of left adrenal gland was bigger than the right one in one case in the treatment group, and the partial light yellow discoloration in the liver, enlargements of the kidneys, adrenal glands and spleen, paleness of the adrenal glands and kidneys were observed in one case each and the partial red discoloration in the thymus was observed in three cases in the negative control group. In females, the retention of clear fluid in the uterus was observed in two cases each and the partial yellow discoloration in the liver was observed in four and one cases in the treatment group and negative control group respectively, and the raised region in the median lobe in the liver was observed in one case in the treatment group, and the red discoloration of caudated lobe in the liver was observed in one case in the negative control group (Table 7).

Table 3. Hematological data of rat treated orally with OCV for 6 weeks (3 times, once every 2 week)

Tests	Units	Groups (dose/head/day)			
		Male		Female	
		G1 (0)	G2 (× 240 ^a)	G1 (0)	G2 (× 240 ^a)
RBC	10 ⁶ /μl	8.72 ± 0.26 ^b	8.88 ± 0.18	8.21 ± 0.20	8.22 ± 0.23
HGB	g/dl	15.3 ± 0.4	15.8 ± 0.3**	14.6 ± 0.4	14.7 ± 0.4
HCT	%	47.2 ± 0.8	48.9 ± 0.8**	44.9 ± 1.2	45.4 ± 1.1
MCV	fl	54.2 ± 1.4	55.1 ± 1.3	54.6 ± 0.9	55.2 ± 1.1
MCH	pg	17.5 ± 0.5	17.8 ± 0.4	17.8 ± 0.2	17.9 ± 0.3
MCHC	g/dl	32.4 ± 0.6	32.4 ± 0.4	32.6 ± 0.3	32.5 ± 0.3
RDW	%	10.8 ± 0.3	11.0 ± 0.3	10.8 ± 0.2	10.6 ± 0.3
HDW	g/dl	2.60 ± 0.18	2.52 ± 0.13	2.45 ± 0.08	2.41 ± 0.19
RET	%	2.32 ± 0.33	2.38 ± 0.30	2.41 ± 0.45	2.44 ± 0.39
PLT	10 ³ /μl	1096.7 ± 106.7	1015.9 ± 112.7	1104.3 ± 69.5	1080.3 ± 114.0
MPV	fl	7.73 ± 0.37	7.41 ± 0.42	7.03 ± 0.89	7.07 ± 0.36
WBC	10 ³ /μl	10.28 ± 2.17	9.78 ± 2.20	4.56 ± 1.54	4.58 ± 1.73
NEU	%	11.49 ± 3.80	12.84 ± 4.84	11.33 ± 2.93	10.29 ± 2.60
LYM	%	82.9 ± 4.0	82.1 ± 5.8	82.9 ± 3.1	84.2 ± 3.0
MONO	%	3.83 ± 0.70	3.43 ± 0.96	3.04 ± 0.91	3.03 ± 0.87
EOS	%	0.89 ± 0.28	0.75 ± 0.25	1.81 ± 0.60	1.45 ± 0.46
BASO	%	0.22 ± 0.08	0.19 ± 0.07	0.10 ± 0.08	0.09 ± 0.07
LUC	%	0.71 ± 0.17	0.70 ± 0.18	0.77 ± 0.15	0.95 ± 0.30
PT	sec	9.53 ± 1.00	9.53 ± 0.37	9.58 ± 0.28	9.47 ± 0.35
APTT	sec	18.2 ± 0.5	17.8 ± 0.6	15.9 ± 1.4	15.8 ± 1.3

** A significant difference at $p < 0.01$ level compared with the negative control.

^a) ×AHD (anticipated human dose).

^b) Data are expressed as Mean ± S.D.

Table 4. Clinical biochemistry data of rat treated orally with OCV for 6 weeks (3 times, once every 2 week)

Clinical Biochemistry Test					
Tests	Units	Groups (dose/head/day)			
		Male		Female	
		G1 (0)	G2 ($\times 240^{\text{a}}$)	G1 (0)	G2 ($\times 240^{\text{a}}$)
AST	U/L	85.6 \pm 15.3 ^{b)}	81.2 \pm 15.2	88.6 \pm 9.9	85.2 \pm 18.1
ALT	U/L	41.9 \pm 7.9	45.2 \pm 10.0	34.4 \pm 5.3	36.0 \pm 5.5
ALP	U/L	106.3 \pm 15.6	106.8 \pm 18.5	71.4 \pm 14.0	70.8 \pm 14.4
CPK	U/L	152.4 \pm 57.4	141.4 \pm 32.8	148.6 \pm 40.3	105.8 \pm 56.8
TBIL	mg/dl	0.17 \pm 0.02	0.17 \pm 0.01	0.18 \pm 0.02	0.19 \pm 0.02
GLU	mg/dl	155.2 \pm 25.3	164.5 \pm 14.5	108.7 \pm 13.9	111.9 \pm 9.4
TCHO	mg/dl	97.6 \pm 17.9	107.5 \pm 14.8	91.0 \pm 14.5	105.0 \pm 20.1
TG	mg/dl	52.2 \pm 16.1	50.5 \pm 10.9	31.5 \pm 6.5	31.5 \pm 5.8
TP	g/dl	6.11 \pm 0.12	6.23 \pm 0.19	5.89 \pm 0.19	5.97 \pm 0.27
ALB	g/dl	3.05 \pm 0.08	3.10 \pm 0.13	3.15 \pm 0.11	3.19 \pm 0.16
A/G	ratio	1.00 \pm 0.06	0.99 \pm 0.04	1.15 \pm 0.08	1.15 \pm 0.05
BUN	mg/dl	18.7 \pm 2.0	18.9 \pm 2.4	21.8 \pm 2.2	20.2 \pm 2.2
CRE	mg/dl	0.45 \pm 0.03	0.45 \pm 0.04	0.50 \pm 0.03	0.47 \pm 0.03*
IP	mg/dl	6.92 \pm 0.38	6.83 \pm 0.35	6.64 \pm 0.58	6.42 \pm 0.62
Ca ²⁺	mg/dl	9.57 \pm 0.18	9.42 \pm 0.25	9.21 \pm 0.40	9.29 \pm 0.29
Na ⁺	mmol/L	140.5 \pm 0.7	140.2 \pm 0.8	139.8 \pm 0.6	140.0 \pm 0.6
K ⁺	mmol/L	4.65 \pm 0.33	4.83 \pm 0.22	4.16 \pm 0.32	4.33 \pm 0.35
Cl ⁻	mmol/L	103.0 \pm 2.6	104.3 \pm 0.9	105.4 \pm 0.7	105.3 \pm 0.9

* A significant difference at $p < 0.05$ level compared with the negative control.

^{a)} \times AHD (anticipated human dose).

^{b)}Data are expressed as Mean \pm S.D.

Histopathological examination. The incidence of abnormalities was not significantly different to that in control group. No significant changes were observed in the treatment group compared to the negative control group and other spontaneous changes³⁾ were observed in low frequency. Among abnormal gross findings observed at necropsy, the red discoloration in the thymus was identified as agonal congestion/hemorrhage, and the red discoloration of caudate lobe in the liver as necrosis of caudate lobe, the raised region in the median lobe as hepatodiaphragmatic nodule and the retention of clear fluid in the uterus as hydrometra. No unusual findings were observed in the adrenal glands and kidneys (Table 8).

DISCUSSION

The present study was performed to evaluate the toxicity and to identify the target organ after repeated oral administration of Oral Cholera Vaccine in Sprague-Dawley rats for 6 weeks (3 times, once every 2 week).

Test article were administered to female and male Sprague-Dawley rats at does level of 240 times the anticipated human dose or OCV Placebo (negative control) by oral gavage. The parameters including changes in clinical signs, body weights, food and water consumptions, urinalysis, hematological and clinical biochemistry tests, organ weights, necropsy and histopathological examination were observed.

In this study we could not find any changes in mortality, clinical signs and change of body weight, water and food consumption except for a significant ($p < 0.05$) decrease of the weight gain and the fasting weight at necropsy in the male treatment group and consumption of food and water in the female treatment group on week 4. In urinalysis, hematological test and clinical biochemistry test, there were no significant change in present study except for the significant ($p < 0.01$) increases of HGB and HCT in the male treatment group and the significant ($p < 0.05$) decrease of CRE in female treatment group. In addition, no meaningful change in organ weight, necropsy findings and histopathological examination were observed. However, significant ($p < 0.01$) changes of the absolute and relative weights of the epididymides in male treatment group and significant ($p < 0.05$) changes of the relative weight of heart and liver in the female test treatment group were observed.

The significant ($p < 0.05$) decreases of the weight gain and the fasting weight at necropsy observed in the male treatment group showed a small difference compared to the negative control group. But they were considered as not meaningful change, because these changes were not observed in females. Besides the weight gain and body weight in this present study were corresponded to same strain and aged normal rats as previously reported (Klinger *et al.*, 1996). Therefore these changes were not related to the administration of test article. Also the decreases of food and water

Table 5. Absolute and relative organ weights of male rats treated orally with OCV for 6 weeks (3 times, once every 2 week)

Parameters	Groups (dose/head/day)	
	G1 (0)	G2 ($\times 240^a$)
BODY WEIGHTS (g) ^b	366.60 \pm 13.80 ^c	352.52 \pm 15.40*
ADRENAL GLAND-LEFT % to BODY WEIGHT	0.0286 \pm 0.0088 0.0078 \pm 0.0024	0.0293 \pm 0.0112 0.0083 \pm 0.0028
ADRENAL GLAND-RIGHT % to BODY WEIGHT	0.0291 \pm 0.0075 0.0079 \pm 0.0021	0.0255 \pm 0.0032 0.0072 \pm 0.0010
PITUITARY GLAND % BODY WEIGHT	0.0121 \pm 0.0009 0.0033 \pm 0.0002	0.0115 \pm 0.0012 0.0032 \pm 0.0003
THYMUS % to BODY WEIGHT	0.4541 \pm 0.0821 0.1242 \pm 0.0237	0.4034 \pm 0.0742 0.1147 \pm 0.0217
PROSTATE GLAND % to BODY WEIGHT	0.4826 \pm 0.0825 0.1318 \pm 0.0238	0.5180 \pm 0.1176 0.1465 \pm 0.0309
TESTIS-LEFT % to BODY WEIGHT	1.9699 \pm 0.1187 0.5374 \pm 0.0260	1.9647 \pm 0.1410 0.5573 \pm 0.0304
TESTIS-RIGHT % to BODY WEIGHT	1.9354 \pm 0.1250 0.5282 \pm 0.0330	1.9660 \pm 0.1434 0.5576 \pm 0.0297
EPIDIDYMIS-LEFT % to BODY WEIGHT	0.5895 \pm 0.0370 0.1609 \pm 0.0103	0.6187 \pm 0.0357 0.1756 \pm 0.0079**
EPIDIDYMIS-RIGHT % to BODY WEIGHT	0.5887 \pm 0.0352 0.1609 \pm 0.0129	0.6372 \pm 0.0382** 0.1809 \pm 0.0099**
SPLEEN % to BODY WEIGHT	0.7884 \pm 0.1696 0.2150 \pm 0.0458	0.7118 \pm 0.0837 0.2018 \pm 0.0211
KIDNEY-LEFT % to BODY WEIGHT	1.3445 \pm 0.1550 0.3668 \pm 0.0409	1.2944 \pm 0.1188 0.3672 \pm 0.0299
KIDNEY-RIGHT % to BODY WEIGHT	1.3337 \pm 0.1149 0.3640 \pm 0.0310	1.3148 \pm 0.1105 0.3727 \pm 0.0222
HEART % to BODY WEIGHT	1.2458 \pm 0.1050 0.3399 \pm 0.0279	1.2311 \pm 0.0707 0.3492 \pm 0.0126
LUNG % to BODY WEIGHT	1.6090 \pm 0.1336 0.4392 \pm 0.0372	1.5593 \pm 0.1377 0.4426 \pm 0.0381
BRAIN % to BODY WEIGHT	1.9450 \pm 0.1201 0.5306 \pm 0.0262	1.9289 \pm 0.1350 0.5470 \pm 0.0244
LIVER % to BODY WEIGHT	10.5661 \pm 0.8297 2.8817 \pm 0.1911	9.8745 \pm 0.6346 2.8005 \pm 0.1167

*/** A Significant difference at $p < 0.05/p < 0.01$ levels compared with the negative control.

^a \times AHD (anticipated human dose).

^bThe body weights were measured right before necropsy after overnight fasting.

^cData are expressed as Mean \pm S.D.

consumption in the female treatment group on week 4 were not attributable to the test article because the symptoms were observed temporarily, and they did not accompany the related observation item such as body weights. The decreases of food and water consumption may be due to incomplete water supply in some cages.

Table 6. Absolute and relative organ weights of female rats treated orally with OCV for 6 weeks (3 times, once every 2 week)

Parameters	Groups (dose/head/day)	
	G1 (0)	G2 ($\times 240^a$)
BODY WEIGHTS (g) ^b	206.81 \pm 14.49 ^c	215.12 \pm 6.95
OVARY-LEFT % to BODY WEIGHT	0.0417 \pm 0.0077 0.0201 \pm 0.0031	0.0409 \pm 0.0040 0.0190 \pm 0.0015
OVARY-RIGHT % to BODY WEIGHT	0.0419 \pm 0.0045 0.0203 \pm 0.0016	0.0425 \pm 0.0057 0.0198 \pm 0.0029
ADRENAL GLAND-LEFT % to BODY WEIGHT	0.0287 \pm 0.0037 0.0139 \pm 0.0010	0.0289 \pm 0.0031 0.0134 \pm 0.0014
ADRENAL GLAND-RIGHT % to BODY WEIGHT	0.0297 \pm 0.0037 0.0143 \pm 0.0010	0.0290 \pm 0.0032 0.0134 \pm 0.0013
PITUITARY GLAND % BODY WEIGHT	0.0118 \pm 0.0010 0.0057 \pm 0.0006	0.0124 \pm 0.0010 0.0057 \pm 0.0005
THYMUS % to BODY WEIGHT	0.2767 \pm 0.0362 0.1341 \pm 0.0176	0.2939 \pm 0.0447 0.1366 \pm 0.0197
UTERUS % to BODY WEIGHT	0.5176 \pm 0.2319 0.2528 \pm 0.1203	0.5664 \pm 0.1908 0.2638 \pm 0.0897
SPLEEN % to BODY WEIGHT	0.5334 \pm 0.0520 0.2577 \pm 0.0132	0.5750 \pm 0.0565 0.2677 \pm 0.0297
KIDNEY-LEFT % to BODY WEIGHT	0.7353 \pm 0.0493 0.3561 \pm 0.0205	0.7474 \pm 0.0540 0.3473 \pm 0.0211
KIDNEY-RIGHT % to BODY WEIGHT	0.7459 \pm 0.0712 0.3607 \pm 0.0217	0.7734 \pm 0.0776 0.3593 \pm 0.0326
HEART % to BODY WEIGHT	0.7784 \pm 0.0823 0.3763 \pm 0.0282	0.8487 \pm 0.0556 0.3945 \pm 0.0210*
LUNG % to BODY WEIGHT	1.1759 \pm 0.1033 0.5697 \pm 0.0505	1.1664 \pm 0.0699 0.5426 \pm 0.0346
BRAIN % to BODY WEIGHT	1.7542 \pm 0.0681 0.8530 \pm 0.0820	1.7107 \pm 0.0520 0.7959 \pm 0.0316
LIVER % to BODY WEIGHT	5.2542 \pm 0.3697 2.5419 \pm 0.0887	5.6056 \pm 0.2542* 2.6073 \pm 0.1254

* A Significant difference at $p < 0.05$ level compared with the vehicle control.

^a \times AHD (anticipated human dose).

^bThe body weights were measured right before necropsy after overnight fasting.

^cData are expressed as Mean \pm S.D.

The significant ($p < 0.01$) increases of HGB and HCT were observed in the male treatment group. Sometimes, these hematological changes occur by dehydration related loss of fluids (Morris, 1982). However, clinical signs such as weight loss, diarrhea and parameter changes related dehydration were not observed. In addition, changes of HGB and HCT were found within the normal range. Similarly, the significant ($p < 0.05$) decrease of CRE in female treated group were not accompanied with any significant changes in the related parameters and were found within the normal

Table 7. Macroscopic findings of rats treated orally with OCV for 6 weeks (3 times, once every 2 week)

Organs	Findings	Groups (dose/head/day)	
		G1 (0)	G2 ($\times 240^{\text{a}}$)
			Male
Thymus	Red discoloration, partially	3	0
Liver	Light yellow discoloration, partially	1	0
Spleen	Enlargement	1	0
Adrenal gland	Enlargement & Paleness	1	0
	Left side bigger than the right	0	1
Kidney	Enlargement & Paleness	1	0
			Female
Uterus	Retention of clear fluid	2	2
Liver	Light yellow discoloration, partially	1	4
	Red discoloration in caudate lobe	1	0
	Raised region (1 ea)	0	1

^{a)} \times AHD (anticipated human dose).

Table 8. Histopathological findings of rats treated orally with OCV for 6 weeks (3 times, once every 2 week)

Organs	Findings	Groups (dose/head/day)	
		G1 (0)	G2 ($\times 240^{\text{a}}$)
			Male
PROSTATE GLAND	Infiltration, mononuclear cell	5	4
	Nephropathy, chronic progressive	1	3
KIDNEY	Cast, hyaline	4	0
	Basophilia, tubule	2	2
	Mineralization, cortex	1	0
LIVER	Infiltration, mononuclear cell	3	0
SPLEEN	Hematopoiesis, extramedullary	2	2
LUNG	Infiltration, mononuclear cell	4	4
THYMUS	Congestion/hemorrhage, agonal	2	0
THYROID GLAND	Cyst	1	0
	Ectopia, thymus	1	1
			Female
ADRENAL GLAND	Vacuolated foci	1	0
UTERUS, DUPLEX (BODY & HORN)	Hydrometra	2	0
KIDNEY	Nephropathy, chronic progressive	1	2
	Infiltration, mononuclear cell	2	0
	Cast, hyaline	0	2
	Basophilia, tubule	0	2
	Mineralization, cortex	0	2
LIVER	Infiltration, mononuclear cell	1	2
	Hepatodiaphragmatic nodule	0	1
	Necrosis, caudate lobe	1	0
SPLEEN	Hematopoiesis, extramedullary	2	0
LUNG	Infiltration, mononuclear cell	2	0
THYMUS	Hyperplasia, epithelial	2	0
THYROID GLAND	Cyst	0	1
COLON	Distension	1	0
RECTUM	Distension	1	3
HARDERIAN GLAND	Infiltration, mononuclear cell	1	0

^{a)} \times AHD (anticipated human dose).

range (Han *et al.*, 2010).

The increases in the absolute or relative weights of right and left epididymis observed in the male treatment group and the increases in the relative weight of the heart and absolute weight of the liver observed in the female treatment group were changes within the normal range (Sifontes-Rodríguez *et al.*, 2009) and no abnormal histopathological findings were observed in these organs.

The partial light yellow discoloration of liver, the partial red discoloration of thymus, enlargements of the kidneys, adrenal glands and spleen, paleness of the adrenal glands and kidneys, the retention of clear fluid in the uterus, the raised region in the median lobe and the red discoloration of caudated lobe of liver detected as gross findings and hepatodiaphragmatic nodule, hydrometra, agonal congestion/hemorrhage detected as histopathological findings were considered as accidental or spontaneous lesions because they were restricted in some individual cases and also observed in negative control group (Greaves, 2000). Thus, these changes were not judged to the administration of test article.

Taken together when the test article, Oral Cholera Vaccine, was orally administered 3 times for 6 weeks (once every 2 weeks) in Sprague-Dawley male and female rats at a dose equivalent to 240 times to the anticipated human dose, no toxicologically significant changes related to the treatment were observed in all test parameters and the target organs were not established.

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